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The overall goal of our laboratory is to elucidate the mechanism of signal transduction and transcriptional regulation by the estrogen receptor (ER), a ligand-regulated transcription factor that plays a critical role in the development, progression and hormone responsiveness of breast cancer cells. Combining genetic, molecular and biochemical approaches, we have identified the Hsp90-associated co-chaperone, p23, as a key regulator of ER action and have also determined that ER is phosphorylated and regulated by the cyclin A/Cdk2 complex. Understanding of the communication between ER and these regulator factors is fundamental to understanding the mechanism of ER-regulated gene expression and may reveal novel points of intervention to be exploited in the development of new therapies for breast cancer.

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5. Introduction:

The long-term objective of this proposal is to elucidate the molecular mechanisms of signal transduction and transcriptional regulation by the estrogen receptor (ER), a hormone-dependent transcriptional regulatory protein involved in the initiation and maintenance of many human breast tumors. At present we are characterizing the regulation of ER activity by the co-chaperone p23 as well as identifying proteins that interact with ER in a phosphorylation-dependent manner.

6. Body:

Research Accomplishments:

1. Regulation of ER activity by molecular chaperones

Using dosage suppression analysis, we have isolated the yeast homologue of the human p23, a component of the Hsp90-based molecular chaperone complex, as a factor that regulates ER ligand-dependent and independent activity. *(See Knoblauch, R. and Garabedian, M.J., (1999) Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, Mol. Cell. Biol. 19, 3738-3759).* Future studies include a structure function analysis of the

p23:ER interaction and targeted deletion of p23 from the mouse genome and its participation in ER signaling *in vivo*.

2. Regulation of ER transcriptional activation by phosphorylation

Genetic, molecular and biochemical strategies are also being used to define the effects of phosphorylation on ER function. We have previously shown that activation of the cyclin A/Cdk2 complex increases hormone-independent and -dependent transcriptional activation by the ER in cultured mammalian cells. We have recently identified serines 104 and 106 of human ER as the likely targets of cyclin A/Cdk2 phosphorylation *(see Rogatsky, I., Trowbridge, J.M. and Garabedian, M.J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A/Cdk2 complex, J. Biol. Chem. 274, 22296-22302).* We proposed that ER phosphorylation by cyclinA/Cdk2 provides sites that recruit additional proteins from binding to ER, thus affecting transcriptional activation by the receptor. We are currently identifying factors that interact with ER in a phosphorylation-dependent manner using a novel screening

method developed in our lab *(see Hittelman, A.B., Burakov, D., Iñiguez-Lluhí, J.A., Freedman, L.P., and Garabedian, M.J., (1999) Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins, EMBO J 18.* 5380-5388).

In view of increasing clinical data linking Cdk dysregulation to a variety of human cancers, notably breast cancer, we believe that the subversion of the cyclin A/Cdk2 pathway might account for a subpopulation of breast hyperplasias and/or tumors, a hypothesis we are also testing.

With nearly half of all human breast cancers dependent upon estrogen for growth, identifying the proteins that are recruited to ER in a phosphorylation-dependent manner and understanding how molecular chaperones function in ER signaling may provide valuable targets for inhibiting ER action and stopping cancer growth.

7. Appendices:

1. Key research accomplishments:

- Identified p23 as a key regulator of estrogen receptor signaling.
- Identified the sites of estrogen receptor phosphorylation by the cyclin A-Cdk2 complex.
- Developed methodologies to identify proteins that interact with the estrogen receptor transcriptional regulatory regions.

2. Reportable outcomes:

Knoblauch, R. and Garabedian, M.J., (1999) Role of Hsp-90-associated cochaperone p23 in estrogen receptor signal transduction, *Mol. Cell. Biol.* 19, 3738-3759

Rogatsky, I., Trowbridge, J.M. and Garabedian, M.J. (1999) Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A/Cdk2 complex, *J. Biol. Chem.* 274, 22296-22302.

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3. A copy of each manuscripts is enclosed.

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Role for Hsp90-Associated Cochaperone p23 in Estrogen Receptor Signal Transduction

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The mechanism of signal transduction by the estrogen receptor (ER) is complex and not fully understood. In addition to the ER, a number of accessory proteins are apparently required to efficiently transduce the steroid hormone signal. In the absence of estradiol, the ER, like other steroid receptors, is complexed with Hsp90 and other molecular chaperone components, including an immunophilin, and p23. This Hsp90-based chaperone complex is thought to repress the ER's transcriptional regulatory activities while maintaining the receptor in a conformation that is competent for high-affinity steroid binding. However, a role for p23 in ER signal transduction has not been demonstrated. Using a mutant ER (G400V) with decreased hormone binding capacity as a substrate in a dosage suppression screen in yeast cells (*Saccharomyces cerevisiae*), we identified the yeast homologue of the human p23 protein (yhp23) as a positive regulator of ER function. Overexpression of yhp23 in yeast cells increases ER transcriptional activation by increasing estradiol binding in vivo. Importantly, the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of both ER and estradiol in the cell. Under conditions of high ER expression, ER transcriptional activity is largely independent of yhp23, whereas at low levels of ER expression, ER transcriptional activation is primarily dependent on yhp23. The same relationship holds for estradiol levels. We further demonstrate that yhp23 colocalizes with the ER in vivo. Using a yhp23-green fluorescent protein fusion protein, we observed a redistribution of yhp23 from the cytoplasm to the nucleus upon coexpression with ER. This nuclear localization of yhp23 was reversed by the addition of estradiol, a finding consistent with yhp23's proposed role as part of the aporeceptor complex. Expression of human p23 in yeast partially complements the loss of yhp23 function with respect to ER signaling. Finally, ectopic expression of human p23 in MCF-7 breast cancer cells increases both hormone-dependent and hormone-independent transcriptional activation by the ER. Together, these results strongly suggest that p23 plays an important role in ER signal transduction.

Estrogen is a steroid hormone responsible for the proper function of a variety of mammalian physiological processes. In addition to its central role in reproduction (6, 33), estrogen also affects the cardiovascular (19), skeletal (47), immune (9), and nervous (57) systems and plays a role in the initiation and progression of breast cancer (58).

The estrogen signal is mediated by the estrogen receptor (ER), a ligand-inducible transcription factor. In the absence of estradiol, the ER is found predominantly in the nucleus (32, 56), as part of a multiprotein complex consisting of a dimer of Hsp90 (6), a p23 monomer (42), and one of several immunophilins, including Cyp-40 (49) and FKBP52 (48). It has been proposed that this Hsp90-based chaperone complex inactivates the ER's transcriptional regulatory capabilities and maintains the ER in a conformation competent for steroid binding (46). Upon binding estradiol, ER dissociates from this complex, dimerizes, and recognizes specific DNA sequences (35), termed estrogen-response elements (EREs), within the promoters of estrogen-responsive genes. Once bound to an ERE, ER is believed to modulate transcription of the linked gene through direct or indirect interactions with general transcriptional factors (15, 25).

Although many aspects of ER signaling are not yet understood, it appears that the proteins essential to ER function are conserved among eukaryotes to such an extent that introduction of ER into *Saccharomyces cerevisiae*, which lacks endogenous ER, is sufficient for the faithful reconstitution of estrogen signaling within these cells (18, 41). When expressed in yeast cells, the human ER will activate transcription from EREs located in reporter gene promoters in response to estradiol. This ability of ER to function within yeast cells allows a wide variety of genetic approaches to be taken toward defining the mechanisms of signal transduction and transcriptional regulation by the receptor.

To identify proteins that affect ER function, we have carried out a dosage suppression screen in yeast cells. In this technique, a mutant ER protein, with a reduced ability to activate transcription, is used as a substrate to isolate a yeast gene product(s) that is capable of overcoming this mutant phenotype by favoring the interaction between ER and these factors, thus reconstituting receptor transcriptional activity. The mutant ER used in our dosage suppression screen contains a glycine-to-valine substitution at position 400 (G400V ER). This mutation is believed to alter the conformation of the ligand-binding domain, resulting in decreased hormone binding by the receptor, with a corresponding reduction in G400V ER's ability to activate transcription in response to estradiol (54). This mutant was selected as the substrate for the screen because it affects an early step in the ER signaling pathway, namely steroid binding, and therefore has the potential to result in the isolation of factors important for either steroid binding or transcriptional activation. We anticipate that characterization of these proteins will ultimately lead to a more complete understanding of the ER signal transduction pathway.

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Using G400V ER as our dosage suppression screen substrate, we have isolated a yeast gene that, when overexpressed, is capable of increasing both G400V and wild-type (wt) ER's ability to activate transcription in response to estradiol. This gene product is the yeast homologue of the vertebrate p23 protein (yhp23) (28), a component of the Hsp90-based molecular chaperone complex associated with unliganded steroid receptors as part of the aporeceptor complex. We have examined the functional relationship between p23 and ER in vivo under a range of receptor, estradiol, and p23 concentrations. Our findings suggest that p23 plays a role in ER signal transduction.

MATERIALS AND METHODS

Yeast strains and growth conditions. The W303a (**a** *ade2 leu2 his3 trp1 ura3*) yeast strain was used in experiments where indicated. The yhp23/SBA1 knockout (KO) and parental (PA) strains are described elsewhere (2). Standard genetic methods were used for growth and manipulation. Cultures were propagated at 30°C in rich medium (yeast extract-peptone-dextrose) or selective minimal medium with 2% glucose (or raffinose or galactose) supplemented with amino acids. To induce gene expression from vectors containing the Gal1-10 inducible promoter, yeast cells were grown in selective medium containing either 2% galactose–1% raffinose or 2% raffinose.

Plasmid constructs. The YKL117w gene was cloned from the 4.3 library plasmid by PCR with the following primers: 5′-GAAGATCTCCACCATGTAC CCATACGATGTTCCTGACTATGCGTCCGATAAAGTTATTAACCCTC AAGTTGC-3′ (encoding a hemagglutin [HA] epitope and containing a *Bgl*II site) and 5′-CCCCATGGTTACTCATTCTAGCACTCCAGGTTGATTT-3′. The PCR product was gel purified and subcloned into the pGEM-T Easy vector (Promega). The entire fragment was sequenced to ensure the fidelity of the PCR product. HA-yhp23 was released from this vector by digesting it with *Bgl*II (within primer) and *Pst*I (within the polylinker of pGEM-T Easy) and then subcloned into *Bgl*II/*Pst*I-digested pCMV5. HA-yhp23 was released from pCMV5 by the *Bgl*II/*Bam*HI digest and subcloned into the *Bam*HI site of the yeast expression vectors containing the glycerol-phosphate dehydrogenase promoter (GPD) pRS314$_{GPD}$(Trp1) and PRS315$_{GPD}$(Leu2). The yhp23-green fluorescent protein (GFP) fusion protein was created by subcloning the *Hin*dIII/*Xba*I fragment of GFP from pEGFP-N3 (Clontech) into a *Bgl*II/*Hin*dIII fragment of HA-yhp23 in the pGEM-T Easy vector and placing the HA-yhp23-GFP fusion protein into pCMV5 at the *Bgl*II/*Xba*I sites. The HA-yhp23-GFP fusion protein was excised from pCMV5 by using *Bgl*II/*Bam*HI and then subcloned into the *Bam*HI site of the yeast expression vector pRS314$_{GPD}$. The ER$_{1-115}$–Lex DNA binding domain fusion protein was constructed by excising the *Eco*RI/*Pvu*II fragment of pGEX-ER$_{1-185}$ (a kind gift from P. Kushner) and inserting it into the *Eco*RI- and *Xho*I-digested pEG 202 two-hybrid vector (20). The *Xho*I sticky end was first blunted to make ligation with the *Pvu*II blunt end possible.

An HA epitope was also placed at the amino terminus of the human p23 cDNA by PCR by using the following primers: 5′-GCGGATCCACCATGTAC CCATACGATGTTCCTGACTATGCGCAGCCTGCTTCTGCAAAGTGGTAC GATCG-3′ (containing a *Bgl*II site and encoding an HA epitope) and 5′-CACCA CCCATGTTGTTCATCATCTCAGAG-3′. The same PCR and cloning strategy was used to create the HA-p23 yeast and mammalian expression vectors.

wt ER, G400V ER, and wt glucocorticoid receptor (GR) were expressed from vectors containing the Gal1-10 promoter, the 2μm plasmid replication origin from yeast cells, and either the *TRP1* (p2T-GAL) or *HIS3* (p2H-GAL) gene. Reporter plasmids ERE-CYC1-LacZ or GRE-CYC1-LacZ contain a single ERE or three glucocorticoid response elements (GREs) upstream of a truncated *CYC1* promoter linked to the β-galactosidase gene; these plasmids also contain the *URA3* gene as a selectable marker and the yeast 2μ plasmid replication origin. Full-length GRIP1 was expressed constitutively in yeast from the alcohol dehydrogenase promoter as described previously (21).

Transient transfections. MCF-7 cells were seeded onto 60-mm dishes at 2.5 × 10^5 cells/dish in phenol red-free Dulbecco modified Eagle medium supplemented with 10% charcoal-stripped fetal bovine serum. The following day the cells were transfected by the liposome-mediated method as described by the manufacturer (Trans IT-100; Pan Vera, Madison, Wis.) with 5 μg of ERE-thymidine kinase-luciferase reporter plasmid (XETL) and 2 μg of pCMV-HA-p23. At 12 h posttransfection, cells were refed with the same medium containing 0.1 nM 17-β-estradiol or ethanol vehicle. A luciferase assay was performed 24 h later as previously described (50).

β-Galactosidase assays. With yeast liquid cultures, quantitative β-galactosidase measurements were carried out as previously described (18). Cultures were grown overnight in selective media containing 2% glucose. Equal numbers of cells (as determined by measuring the optical density at 600 nm [OD_{600}]) were pelleted and washed in sterile water to remove glucose medium and subcultured into 2 ml of selective medium containing either 2% galactose–1% raffinose or 2% raffinose. Steroid hormones were added to the medium as a 1,000-fold dilution in ethanol. Cultures were incubated for 12 h at 30°C. One-half of the cells were pelleted, washed in 0.5 ml of LacZ buffer (5 mM KCl, 0.5 mM $MgSO_4 \cdot 7H_2O$, 60 mM $Na_2HPO_4 \cdot 7H_2O$, 60 mM $NaH_2PO_4 \cdot H_2O$ [pH 7.0], with 0.025% β-mercaptoethanol added freshly), and pelleted again. Cells were resuspended in 50 μl of LacZ buffer and permeabilized by adding 50 μl of $CHCl_3$–20 μl of 0.1% sodium dodecyl sulfate (SDS) and vortexing for 10 s. After 5 min, 0.5 ml of a 2-mg/ml concentration of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was added, and the reaction was allowed to continue for 1 to 10 min, after which the reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3. Reactions were centrifuged for 1 min to pellet the cell debris, and levels of β-galactosidase activity were determined by measuring the OD_{420}. The cell number was determined by measuring the OD_{600} of the remaining cells. Receptor activity is expressed as β-galactosidase units, determined by using the following equation: β-galactosidase units = $(1,000 \times OD_{420})/(\text{reaction volume [milliliters]} \times \text{reaction time [minutes]} \times OD_{600})$. All experiments were performed in triplicate, and the data shown are representative of multiple experiments. The experiments were performed in the linear range of the assay.

Plate assays were performed by replica plating colonies from glucose plates onto galactose X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates containing 1 nM 17-β-estradiol.

Preparation of yeast extracts and immunoblotting. Yeast extracts were prepared from 5-ml cultures. Equal numbers of cells (as determined by measuring the OD_{600}) were pelleted and washed in 0.5 ml of 1× phosphate-buffered saline (PBS) supplemented with 3 mM dithiothreitol and protease inhibitors, including 1 mM phenylmethylsulfonyl fluoride and 1 μg each of aprotinin, pepstatin A, and leupeptin per ml. Subsequent steps were carried out at 4°C. Cells were again pelleted and resuspended in 200 μl of receptor buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 50 mM NaCl; 20% glycerol) containing protease inhibitors. An equal volume of glass beads was added to each tube, and cells were lysed by shaking for 20 min in an Eppendorf horizontal shaker. Extracts were separated from glass beads by centrifuging the extracts through a small hole (made with a 20-gauge needle) at the bottom of the microcentrifuge tube. The lysates were subsequently cleared by centrifugation at 10,000 rpm for 15 min, after which the supernatants were transferred to a new tube. The protein concentration of each extract was measured by using the Bio-Rad Protein Assay and then standardized accordingly. For Western blotting, protein extracts (50 to 100 μg) were fractionated on SDS–10% polyacrylamide gels and transferred to Immobilon paper (Millipore). Blots were probed either with a combination of the ER monoclonal antibodies C311 and C314 (Santa Cruz) or with the anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). Rabbit polyclonal antiserum against endogenous yhp23 was the generous gift of B. Freeman (University of California at San Francisco). The blots were developed with horseradish peroxidase-coupled sheep anti-mouse or donkey anti-rabbit antibodies and enhanced chemiluminescence reagent (Amersham).

In vivo estradiol-binding assay. Ligand-binding assays were carried out as previously described (34). Duplicate cultures of yeast cells containing either G400V ER with or without HA-yhp23 or wt ER with or without HA-yhp23 were inoculated into selective liquid medium containing 2% galactose–1% raffinose and grown overnight at 30°C to induce receptor expression. Equal numbers of cells were pelleted and inoculated into 3 ml of 2% galactose–1% raffinose medium containing either 1 nM (G400V ER) or 0.1 nM (wt ER) of ^{3}H-labeled 17-β-estradiol (72 Ci/mmol) (NET317; NEN) such that the final OD_{600} was 2. Cells were incubated with the labeled estradiol for 1 h at 30°C, after which 1 ml of the cultures was pelleted and washed three times with 2% glucose in PBS, resuspended in 150 μl of the same solution, and then transferred to a scintillation vial. After the addition of scintillation fluid, the total ^{3}H-labeled 17-β-estradiol was measured by scintillation counting. To account for any nonspecific binding, ^{3}H-labeled 17-β-estradiol binding to a GR-containing strain was subtracted from the counts obtained with the ER-expressing strain. β-Galactosidase assays were carried out as described above with 1 ml of the remaining culture.

Subcellular localization of yhp23. W303a was transformed with the pRS314$_{GPD}$-yhp23-GFP expression vector; with the ERE-dependent β-galactosidase reporter plasmid; or with the p2H GAL expression vector containing G400V ER, wt ER, GR, or p2H GAL plasmid alone. Yeast strains were grown in 2 ml of 2% galactose–1% raffinose for 12 h. Cells were fixed by adding 240 μl of 37% formaldehyde to each 2-ml culture and incubated at 30°C for 90 min. Cultures were then pelleted by centrifugation and washed three times with 200 μl of solution A (1.2 M sorbitol, 50 mM KPO_4). Cell walls were digested by resuspending cells in 100 μl of solution A plus 0.1% β-mercaptoethanol, 0.02% Glusulase (Dupont), and 5 μg of Zymolase (U.S. Biological) per ml for 1 h at 37°C. Cells were then washed twice in 100 μl of PBS and once in 100 μl of PBS plus 0.1% Nonidet P-40 (NP-40) and then blocked for 2 h in 5% bovine serum albumin in Tris-buffered saline (pH 7.4) at room temperature. Cells were then incubated with 100 μl of the appropriate anti-steroid receptor primary antibody (for ER, a mixture of monoclonal antibodies C311 and C314 [Santa Cruz], and for GR, monoclonal antibody BuGr2 diluted in blocking solution) for 2 h at room temperature. Cells were washed once in 100 μl of PBS, once in 100 μl of PBS plus 0.1% NP-40, and once again in 100 μl of PBS, followed by incubation with goat anti-mouse rhodamine-conjugated secondary antibody (Vector Labs), diluted in blocking solution, for 4 h at room temperature. Secondary antibody was removed by washing the cells twice in 100 μl of PBS and then twice in 100 μl of PBS plus 0.1% NP-40. In order to visualize the nuclei, cells were then incubated

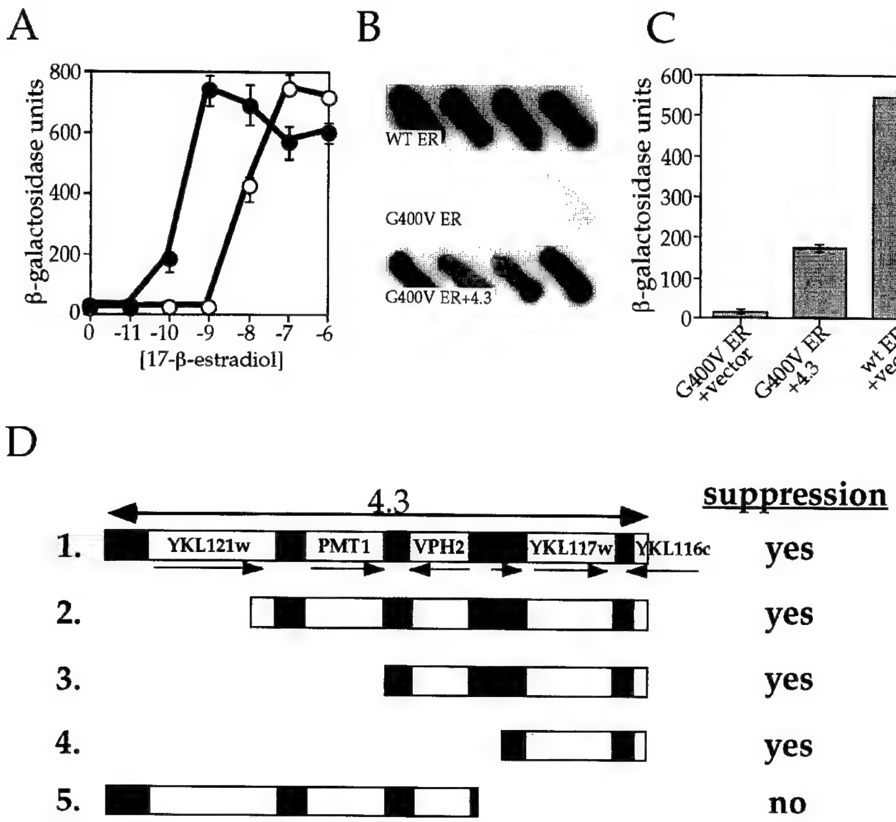

FIG. 1. Isolation of a yeast genomic fragment that suppresses the G400V ER phenotype. (A) Transcriptional activity of wt ER and G400V ER as a function of 17-β-estradiol concentration. The W303a yeast strain was transformed with a galactose-inducible expression vector containing either wt or G400V ER, along with an ERE-containing β-galactosidase reporter plasmid. Transcriptional activation by wt ER (solid circles) and G400V ER (open circles) in response to increasing 17-β-estradiol concentration was determined by liquid β-galactosidase assay as described in Materials and Methods. Note that G400V ER requires a 100-fold-higher estradiol concentration to induce transcriptional activation than wt ER. The dosage suppression screen was carried out in the presence of 1 nM 17-β-estradiol, the conditions under which the G400V ER phenotype is most pronounced. (B) The relative activity of wt ER, G400V ER, and G400V ER plus suppressor 4.3. Four independent colonies on X-Gal indicator plates containing 1 nM 17-β-estradiol are shown and represent wt ER plus empty library plasmid (wt ER), G400V ER plus empty library plasmid (G400V ER), or G400V ER plus suppressor plasmid 4.3 (G400V ER+4.3). (C) Transcriptional activity of wt ER, G400V ER, and G400V ER in the presence of suppressor 4.3. Liquid β-galactosidase assays were performed on yeast strains containing wt ER plus empty library plasmid, G400V ER plus empty library plasmid, or G400V ER plus 4.3 at 1 nM 17-β-estradiol. Suppressor 4.3 increases G400V ER activity 10-fold, bringing its activity to nearly one-third that of wt ER. (D) Identification of YKL117w as the G400V ER suppressor. The sequence of the yeast genomic fragment contained within the suppressor 4.3 was determined by aligning the 5′ and 3′ ends of the insert to the yeast genomic sequence database. Suppressor 4.3 contains an 8,147-bp fragment comprising four complete ORFs (YKL121w, PMT1, VPH2, and YKL117w), a partial ORF (YKL116w), and a tRNA-Ala gene (shaded box). The relative positions and orientations of the genes within the 4.3 fragment are shown schematically (fragment 1). Identification of the gene responsible for suppressing the G400V ER phenotype was accomplished by constructing 5′ and 3′ deletion derivatives of the 4.3 suppressor (fragments 2 to 5) and assaying their ability to increase G400V ER transcriptional activity at 1 nM 17 β-estradiol. "yes" indicates that the fragment was capable of suppressing the G400V ER phenotype; "no" indicates that the fragment failed to increase G400V ER transcriptional activity. YKL117w was present within the suppressing fragments (fragments 1 to 4) but was absent within the fragment that did not suppress (fragment 5), suggesting that its gene product is responsible for increasing G400V ER transcriptional activation. YKL117w encodes the yeast homologue of the human p23 protein (yhp23).

in 1 μg of Hoechst dye H334211 per ml for 10 min, followed by one wash with 100 μl of PBS. Cells were then resuspended in 30 μl of PBS, 5 μl of which was then plated onto a poly-D-lysine-treated microscope slide and allowed to settle for 10 min before excess fluid was removed by aspiration. Unattached cells were then removed by washing the slide with 20 μl of PBS. The cells were mounted by using 3 μl of Citifluor (Ted Pella, Reading, Calif.), and a coverslip was secured to the slide with rubber cement. GFP, rhodamine, and Hoechst signals were imaged and photographed by using a Zeiss Axioplan 2 microscope.

RESULTS

Yeast dosage suppression screen. To compare wt ER and G400V ER function in yeast cells, we constructed two strains containing a galactose-inducible expression vector, encoding either wt ER or G400V ER, and a reporter plasmid containing an ERE located upstream of the β-galactosidase gene. The transcriptional activities of wt ER and G400V ER, as a function of hormone concentration, were measured. Compared to wt ER, G400V ER requires a 100-fold increase in 17-β-estradiol concentration before receptor transcriptional activation is observed in our yeast assay (Fig. 1A). At saturating ligand concentrations, however, G400V ER is able to reach the same maximal activity as wt ER, suggesting that once the block to steroid binding is overcome, the receptor is able to act as efficiently as wt ER in entering the functional interactions downstream of estradiol binding, including both protein-protein and protein-DNA interactions, that are necessary for transcriptional activation.

The G400V ER phenotype is most apparent at a concentration of 1 nM 17-β-estradiol, where wt ER shows maximal transcriptional response, but G400V ER displays only minimal transcriptional activity (Fig. 1A). Exploiting this phenotypic difference, we carried out a dosage suppression screen to identify yeast proteins that, when overexpressed, would increase the transcriptional activity of G400V ER, thereby suppressing the mutant phenotype. The yeast strain containing G400V ER

and an estrogen-responsive β-galactosidase reporter gene was transformed with a high-copy-number yeast genomic library and assayed for G400V ER transcriptional activity on X-Gal indicator plates containing 1 nM 17-β-estradiol. Under these conditions, yeast colonies expressing the wt ER are blue, while the G400V ER-expressing yeast colonies appear white (Fig. 1B). Blue colonies were considered to be potential suppressor candidates. Five candidate suppressors of the G400V ER phenotype were isolated after screening ~6,000 colonies, which we estimate to represent about one-half of the yeast genome.

Identification of the yeast ORF YKL117w as a suppressor of the G400V ER phenotype. One high-copy-number suppressor plasmid, designated 4.3, was found to increase G400V ER transcriptional activity 10-fold, bringing G400V ER transcriptional activation to one-third the wt ER level at 1 nM 17-β-estradiol (Fig. 1B and C). The 5′ and 3′ ends of the insert of the library plasmid were sequenced and aligned with the yeast genome. In this manner, we were able to identify the suppressor DNA as an 8,147-bp genomic fragment of chromosome XI (26) containing multiple open reading frames (ORFs) (Fig. 1D). To identify the suppressing ORF, a series of deletions were constructed and analyzed for their ability to increase G400V ER transcriptional activity. As seen in Fig. 1D, the suppression of the G400V ER phenotype correlates with the presence of ORF YKL117w.

Possible role of YKL117w in ER signaling. A search of the Swissprot database revealed YKL117w to be the yeast homologue of the human p23 protein (yhp23) (28), a component of the Hsp90-based molecular chaperone complex. During completion of this study, two separate reports (2, 16) characterizing yeast strains with YKL117w deleted have called this gene *SBA1*, reflecting an increased susceptibility of steroid signaling to benzoquinone ansamycin antibiotics. Since the YKL117w gene product's homology to the vertebrate p23 protein was of considerable importance in investigating its role in ER function, we have chosen to refer to it as yhp23 to maintain this emphasis.

Although p23's specific function is not known, in vitro studies suggest that it is crucial to the stability of the aporeceptor complex of unliganded steroid receptors. Removal of p23 greatly reduces the formation of stable aporeceptor complexes of the GR (11, 12, 24) and the progesterone receptor (PR) (29, 30). In addition, in vitro studies have suggested that p23 possesses abilities typical of molecular chaperones, since it is capable of interacting with denatured β-galactosidase, suppressing its aggregation and maintaining it in an intermediate, folding-competent conformation (17). The relative importance of these two aspects of p23 function in ER signaling has not been determined.

Characterization of yhp23's role in ER signaling. Having identified yhp23 as the suppressing ORF, we cloned the YKL117w gene with an amino-terminal HA epitope tag (HA-yhp23) into a yeast expression vector containing a constitutively active GPD promoter. We then established a yeast strain that overexpresses HA-yhp23 in the presence of G400V ER, along with a reporter plasmid containing the β-galactosidase gene under control of an ERE. As seen in Fig. 2A, overexpression of HA-yhp23 increases G400V ER transcriptional activity by 10-fold. Extracts from these yeast strains were prepared and analyzed by immunoblotting with antibodies specific for ER and HA. Figure 2B demonstrates that the overexpression of yhp23 does not affect the level of G400V ER protein and, therefore, the increase in transcriptional activity is not a result of increased receptor expression. The HA-tagged yhp23 protein migrates on an SDS-polyacrylamide gel at approximately 34 kDa. The higher molecular mass of the yeast protein (34 kDa versus the 23-kDa human protein) is expected, as the yhp23 is larger (216 residues) than its human counterpart (160 residues). Further comparison of G400V ER activity in the presence or absence of yhp23 overexpression over a range of hormone concentrations revealed that G400V ER function was also enhanced at 10 nM 17-β-estradiol (Fig. 2C). The 30% increase in G400V ER activity seen at this level of hormone, however, is relatively small compared to the 10-fold increase observed at the 1 nM 17-β-estradiol concentration used in the screen. This pattern continues at even higher hormone concentrations, so that no increase of G400V ER activity by yhp23 is observed at 100 nM concentrations of 17-β-estradiol (Fig. 2C) and 1 mM 17-β-estradiol (data not shown). These findings suggest that yhp23's importance to G400V ER signaling decreases at high estradiol concentrations.

Given p23's proposed chaperone-like activities in vitro, it could be argued that yhp23's interaction with the mutant G400V ER might arise as a function of the receptor's misfolded steroid-binding domain rather than reflect a true role in the ER signaling pathway. To determine whether yhp23 overexpression affects wt ER activity, we constructed an additional yeast strain that overexpresses yhp23 in the presence of wt ER, along with an ERE-controlled β-galactosidase gene reporter plasmid. The effect of yhp23 overexpression upon ER activity was then assayed over a range of hormone concentrations (Fig. 2D). wt ER activity was affected in a manner similar to that of G400V ER, albeit less dramatically. At 0.1 nM 17-β-estradiol, wt ER activity is increased by approximately 70% in the presence of overexpressed yhp23, with no increase observed at the higher concentrations of 1 and 10 nM 17-β-estradiol. In contrast to G400V ER, however, an increase in wt ER activity was observed at 0.01 nM 17-β-estradiol. To more closely examine the effect of yhp23 on wt ER activity at this low hormone concentration, we compared wt ER ligand-independent activity to wt ER activity at 0.01 nM 17-β-estradiol. As seen in Fig. 2E, overexpression of yhp23 increases wt ER ligand-independent activity by approximately twofold and ligand-dependent activation by threefold in the presence of 0.01 nM 17-β-estradiol. These findings suggest that overexpression of yhp23 greatly increases wt ER's ability to respond to low levels of ligand. Without yhp23 overexpression, the fold induction of wt ER activity was only 50% upon administration of 0.01 nM 17-β-estradiol compared to a 250% induction of wt ER activity in the presence of overexpressed yhp23. These results suggest that increased levels of yhp23 can facilitate wt ER ligand-independent activity and potentiate ER signaling at low levels of hormone. Thus, the ability of yhp23 to functionally interact with wt ER and not just the G400V ER mutant strongly implicates yhp23 as a member of the ER signaling pathway in yeast cells.

Importantly, yhp23 does not appear to have any effect upon the activity of the ER AF-1 domain. When we fused the amino-terminal 115 residues of ER to a Lex DNA binding domain and expressed this construct in yeast cells, we observed constitutive activation of a Lex-responsive β-galactosidase reporter plasmid, a result consistent with earlier findings demonstrating that the AF-1 activity of ER is contained within this region of the receptor (39). As shown in Fig. 2F, this constitutive activation was not affected by yhp23 overexpression, suggesting that the increased function of both the mutant and the wt ER observed in Fig. 2C and D, respectively, does not appear to be mediated by an increase in AF-1 activity. This finding, taken together with the observed hormone concentration-dependent effect of yhp23 overexpression (Fig. 2C and D), suggests that yhp23 affects the ER signal transduction pathway at the step of ligand binding.

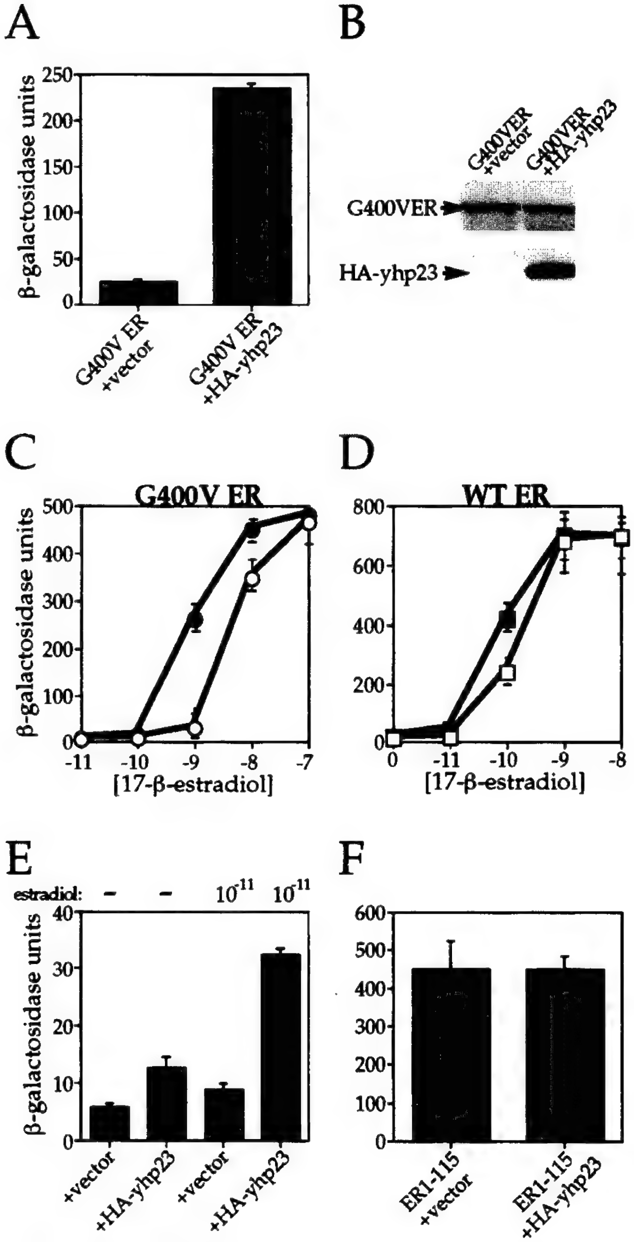

FIG. 2. Effect of yhp23 overexpression on G400V ER and wt ER transcriptional activity. An HA-tagged yhp23 gene was cloned into the yeast expression vector pRS314 downstream of the GPD promoter. Yeast strains were then constructed which coexpress G400V ER or wt ER with the HA-tagged yhp23 in the presence of a β-galactosidase reporter gene under the control of the appropriate hormone response element (ERE). (A) Overexpression of HA-yhp23 increases G400V ER transcriptional activation. The activity of G400V ER in the presence or absence of overexpressed yhp23 was determined by liquid β-galactosidase assay at a 1 nM concentration of 17-β-estradiol. Overexpression of yhp23 increased G400V ER activity approximately 10-fold. (B) Increased transcriptional activation of G400V ER by yhp23 is not a function of increased ER levels. Whole-cell lysates were prepared from the yeast strains described in panel A. Equal amounts of proteins were separated on an SDS–4 to 20% gradient polyacrylamide gel, transferred to Immobilon paper, and probed with an ER-specific monoclonal antiserum (top panel) or a monoclonal antibody directed against the HA epitope on yhp23 (bottom panel) and visualized by enhanced chemiluminescence. (C) Dose-response curves for G400V ER. The activity of G400V ER in the absence (open circles) or presence (solid circles) of overexpressed yhp23 at the indicated 17-β-estradiol concentrations is shown. The increased transcriptional activity displayed by G400V ER in the presence of HA-yhp23 overexpression is seen to be greatest at low hormone concentrations and is lost completely at the highest hormone concentrations assayed. (D) Dose-response curves for wt ER. The activity of wt ER in the absence (open squares) or presence (solid squares) of overexpressed HA-yhp23 at the indicated 17-β-estradiol concentrations is shown. The effect of HA-yhp23 overexpression is seen to be greatest at low hormone concentrations and is lost completely at the highest hormone concentrations assayed. (E) Overexpression of HA-yhp23 increases the ligand-independent activity of the ER. The activity of the ER in the absence or presence of 0.01 nM 17-β-estradiol is shown. Ligand-independent transcriptional activity by ER is approximately threefold higher in the presence of HA-yhp23 overexpression. The addition of 0.01 nM 17-β-estradiol resulted in a greater level of ER ligand-dependent activity in the HA-yhp23 overexpressing strain, suggesting that yhp23 overexpression increases the sensitivity of ER to low concentrations of 17-β-estradiol. (F) HA-yhp23 overexpression does not affect the AF-1 activity of ER. The amino-terminal 115 residues of ER, containing the AF-1 activity of the ER in yeast cells, were fused to a Lex DNA binding domain (see Materials and Methods). Constitutive AF-1 activity resulted in transcription of a reporter β-galactosidase gene downstream of a LexA binding site, which was unaffected by overexpression of HA-yhp23.

yhp23 overexpression increases ligand binding by both G400V ER and wt ER. Considering p23's proposed role in aporeceptor complex formation and given the nature of the G400V ER mutation, we examined whether the increase in G400V ER transcription in the presence of overexpressed yhp23 results from increased estradiol binding by the receptor. Estradiol binding by G400V ER and wt ER in the presence or absence of yhp23 overexpression was measured in vivo. The yeast strains were incubated for 1 h in medium containing ^{3}H-labeled 17-β-estradiol and washed three times to remove unbound steroid, and the amount of estradiol bound to G400V ER and wt ER was measured by quantifying the ^{3}H-labeled 17-β-estradiol content by liquid scintillation counting. β-Galactosidase assays were carried out in parallel on the same cells to correlate effects of ligand binding to transcriptional activation.

We first examined ligand binding by G400V ER at a 1 nM concentration of ^{3}H-labeled 17-β-estradiol. As seen in Fig. 3A, ligand binding by G400V ER was increased by approximately fivefold in the presence of overexpressed yhp23. This increase in ligand binding was found to correlate with a sevenfold increase in G400V ER transcriptional activity (Fig. 3B). Immunoblot analysis showed that the levels of G400V ER were unchanged by yhp23 overexpression (data not shown).

We then examined ligand binding by wt ER, which was also found to increase in the presence of yhp23 overexpression, although to a lesser extent. At a 0.1 nM concentration of ^{3}H-labeled 17-β-estradiol, wt ER ligand binding was increased by approximately 50% (Fig. 3C). β-Galactosidase assay of these cells demonstrated a corresponding 70% increase in transcriptional activity (Fig. 3D). Again, immunoblot analysis showed no difference in the level of ER expression exhibited by the two strains (data not shown). These results indicate that yhp23 overexpression increases ER transcriptional activity by increasing the total number of ligand-bound receptors.

The magnitude of yhp23's effect on wt ER signaling is a function of wt ER and estradiol concentrations. Having demonstrated that overexpression of yhp23 increases wt ER transcriptional activity, we next asked whether a decrease in yhp23 concentration would reduce wt ER activity. To this end, we compared wt ER transcriptional activity in the yhp23 knockout strain (KO) to that in the parental strain (PA) (2), as well as the parental strain overexpressing HA-yhp23 (PA+HA-yhp23 strain). In this way, we were able to assay wt ER function (i) in the absence of yhp23 (KO), (ii) in the presence of endogenous levels of yhp23 (PA), and (iii) in the presence of both endogenous yhp23 and overexpressed HA-yhp23 (PA+HA-yhp23) (Fig. 4B). A wt ER expression vector and an ERE–β-galactosidase reporter plasmid were introduced into these three yeast strains, and wt ER transcriptional activity was assayed at 0.1 nM 17-β-estradiol. The activity of wt ER within the three different strains at this hormone concentration is shown in Fig.

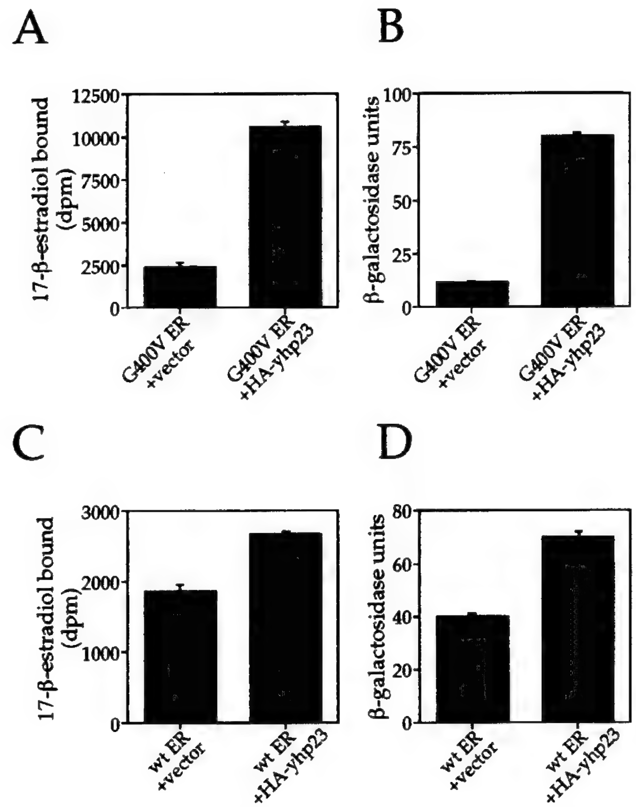

FIG. 3. Overexpression of yhp23 increases estradiol binding by G400V ER and wt ER in vivo. The total amount of estradiol bound by G400V ER and wt ER in the presence or absence of yhp23 overexpression was determined by an in vivo ligand-binding assay with the same yeast strains as described in Fig. 2. After a 1-h incubation in the presence of ^{3}H-labeled 17-β-estradiol, cells were washed to remove unbound ligand, and the amount of bound estradiol was determined by liquid scintillation counting. Liquid β-galactosidase assays were carried out, in parallel, on an aliquot of the same ^{3}H-labeled 17-β-estradiol-incubated cells in order to correlate the levels of ligand binding to the resulting levels of transcriptional activation. Whole-cell extracts from the assayed yeast strains were fractionated on SDS-polyacrylamide gel electrophoresis, and ER expression was examined by use of immunoblotting with an ER-specific rabbit polyclonal antiserum as described in Materials and Methods. No alteration in G400V ER or wt ER levels in response to increased HA-yhp23 expression were noted (data not shown). (A) HA-yhp23 overexpression increases ligand binding by G400V ER. Cells expressing G400V ER in the presence or absence of HA-yhp23 overexpression were assayed for ligand binding in the presence of 1 nM ^{3}H-labeled 17-β-estradiol. Ligand binding by G400V ER is seen to increase approximately fivefold in the presence of overexpressed HA-yhp23. (B) Increased ligand binding by G400V ER in the presence of HA-yhp23 overexpression correlates with an increase in transcriptional activation by the receptor. Liquid β-galactosidase assays, carried out on an aliquot of the cells described in panel A, demonstrate a corresponding sevenfold increase in transcriptional activity by G400V ER. (C) HA-yhp23 overexpression increases ligand binding by wt ER. Cells expressing wt ER in the presence or absence of HA-yhp23 overexpression were assayed for ligand binding in the presence of 0.1 nM ^{3}H-labeled 17-β-estradiol. Ligand binding by wt ER is seen to increase by approximately 50% in the presence of overexpressed HA-yhp23. (D) Increased ligand binding by wt ER in the presence of HA-yhp23 overexpression correlates with an increase in transcriptional activation by the receptor. Liquid β-galactosidase assays, carried out on an aliquot of the cells described in panel C, demonstrate a corresponding 70% increase in transcriptional activity by wt ER.

4A. In the absence of yhp23 (KO), wt ER signaling still occurs, demonstrating that wt ER is capable of functioning in a yhp23-independent manner. Endogenous levels of yhp23 (PA) result in a 50% increase in wt ER activity relative to the KO strain. The PA+HA-yhp23 strain, which contains the highest yhp23 levels (Fig. 4B), exhibits an even higher level of wt ER induction (2.7-fold) than that seen in the KO strain. This increase in

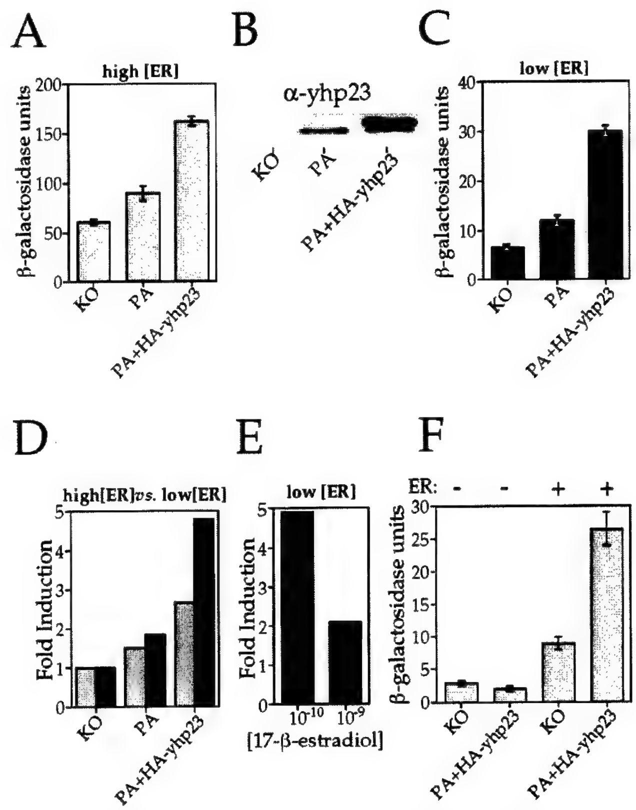

FIG. 4. Yhp23's effect on wt ER activity is a function of the yhp23, ER, and estradiol concentrations. wt ER transcriptional activation was compared in three yeast strains expressing yhp23 at different concentrations. The knockout strain (KO) is yhp23 deficient, the parental strain (PA) expresses yhp23 at endogenous levels, and the HA-yhp23-transformed parental strain (PA+HA-yhp23) expresses endogenous yhp23 and exogenous HA-yhp23. All three strains express wt ER under the control of a galactose-inducible promoter, along with an ERE–β-galactosidase reporter plasmid. wt ER activity in the KO and PA+HA-yhp23 strains was compared over a range of hormone concentrations as shown in Fig. 2D. The greatest effect of yhp23 levels was observed at 0.1 nM 17-β-estradiol. Data shown represent the results of β-galactosidase assays repeated with all three yeast strains (KO, PA, and PA+HA-yhp23) at this hormone concentration. (A) The effect of yhp23 on wt ER transcriptional activation at high ER concentrations. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in galactose-raffinose-supplemented medium containing 0.1 nM 17-β-estradiol. (B) KO, PA, and PA+HA-yhp23 strains express different levels of yhp23. Equal amounts of whole-cell lysates from the KO, PA, and PA+HA-yhp23 strains were analyzed by immunoblotting by using anti-yhp23 polyclonal antibody as described in Materials and Methods. The upper band seen in the PA+HA-yhp23 lane corresponds to the HA-tagged yhp23. (C) The effect of yhp23 on wt ER transcriptional activity at low wt ER concentration. ER transcriptional activity as a function of yhp23 concentration was determined by liquid β-galactosidase assay in cells incubated in raffinose-supplemented medium containing 0.1 nM 17-β-estradiol. Under these conditions ER expression is 10-fold lower than with cells grown in the presence of galactose (not shown). (D) yhp23 induction of wt ER transcriptional activity is inversely proportional to wt ER concentration. The fold induction of yhp23 on ER transcriptional activity at 0.1 nM 17-β-estradiol under conditions of high ER expression (galactose; shaded columns) or low ER expression (raffinose; solid columns) is standardized to the ER activity in the KO strain. (E) The effect of yhp23 on wt ER function is inversely proportional to the ligand concentration. The fold induction of ER transcriptional activation in the PA+HA-yhp23 versus KO strains at 0.1 and 1 nM 17-β-estradiol was determined under conditions of low ER expression (raffinose medium). The results indicate that the fold induction of ER transcriptional activation in the presence of yhp23 overexpression is significantly greater at 0.1 nM 17-β-estradiol than at 1 nM 17-β-estradiol. (F) yhp23 overexpression increases estradiol-independent wt ER transcriptional activation. The effect of yhp23 overexpression on ERE-dependent transcriptional activation in the presence or absence of ER is shown. KO and PA+HA-yhp23 strains containing the ERE reporter construct were transformed with either the empty expression vector or with the ER-containing expression vector and assayed for β-galactosidase activity in galactose-raffinose medium without estradiol.

wt ER activity is not a function of increased receptor levels, since wt ER levels were unchanged in the absence or presence of yhp23 (data not shown). Thus, wt ER transcriptional activity increases in direct proportion to the concentration of yhp23.

Having shown that wt ER activity increases as a function of yhp23 concentration, we next asked if the effect of yhp23 upon ER signaling is also a function of wt ER concentration. This was accomplished by repeating the above experiment in medium containing only raffinose as a carbon source, resulting in low levels of wt ER expression. When wt ER transcriptional activity was assayed under these conditions, a similar pattern of yhp23-dependent ER activation was observed: wt ER functions in the KO strain, and this activity increases as a function of yhp23 concentration (Fig. 4C). Importantly, the magnitude of the effect of yhp23 on wt ER transcriptional activation is greater at the lower wt ER concentration, such that overexpression of the yhp23 in the PA+HA-yhp23 strain results in an almost fivefold increase in wt ER transcriptional activity relative to the KO strain, thereby doubling the induction seen at high wt ER concentrations (Fig. 4D). Thus, the magnitude of yhp23's effect on wt ER transcriptional activity is greater at low, rather than high, wt ER concentrations.

Our earlier studies of yhp23 induction of G400V ER and wt ER activity (Fig. 2C and D) suggested that the importance of yhp23 to wt ER function correlates inversely to hormone concentration. This idea was further confirmed with the KO and PA+HA-yhp23 strains, when the activity of wt ER within these strains was assayed at both 0.1 and 1 nM 17-β-estradiol under conditions of low wt ER expression. As seen in Fig. 4E, the fivefold induction of wt ER transcriptional activity observed within the PA+HA-yhp23 strain at 0.1 nM 17-β-estradiol decreases to only a twofold induction at the higher concentration of 1 nM 17-β-estradiol. Again this trend continues such that, when activities are compared at yet higher hormone concentrations, no difference in activity is observed between the KO and PA+HA-yhp23 strains at 10 nM 17-β-estradiol (data not shown). Thus, the effect of yhp23 overexpression on wt ER transcriptional activation is inversely proportional to the hormone concentration.

Comparison of wt ER activity in the KO and PA+HA-yhp23 strains in the absence of estradiol again confirmed a role for yhp23 in wt ER ligand-independent transcriptional activation. As shown in Fig. 4F, yhp23 is not essential to wt ER-dependent activation in the absence of ligand. Interestingly, as was observed with ligand-dependent activity, wt ER function in the absence of ligand increases with yhp23 overexpression. This effect on transcription is not observed in the absence of wt ER expression, suggesting that yhp23 is operating via wt ER to induce estradiol-independent transcriptional activation.

Thus, the relationship of yhp23 to wt ER signal transduction is dependent not only on concentrations of yhp23 but also upon the levels of wt ER and estradiols. Our data demonstrate that the effect of yhp23 upon wt ER function is most pronounced when wt ER activity is examined at low estradiol and wt ER concentrations.

yhp23 is not essential for the formation of a functional ER-GRIP1 complex. The ER activates transcription in mammalian cells through two transcriptional activation domains, termed AF-1 and AF-2 (36). The AF-1 domain, located within the amino terminus of the receptor, does not require steroid binding to achieve an active conformation (39). In contrast, the AF-2 domain lies within the steroid-binding domain and is dependent upon estradiol binding for its activity (55). The ER AF-2 region has been shown to activate transcription in yeast cells and in cultured mammalian cells through interaction with coactivator proteins, including GRIP1/TIF2 (21) and SRC1 (43). Although AF-2 activity is observed in some promoter contexts in yeast cells (40), the AF-2 coactivators have no yeast homologues, and thus ER transcriptional activity in yeast cells is greatly potentiated in the presence of ectopically expressed mammalian coactivators (22). We therefore sought to more specifically determine whether yhp23 might play a role in AF-2–coactivator interactions.

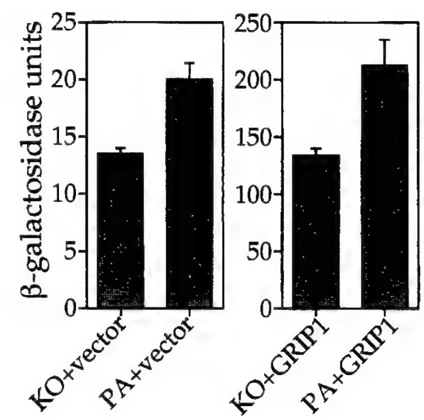

FIG. 5. yhp23 is not essential for the functional interaction between ER and the coactivator GRIP1. yhp23 KO and PA strains were constructed that express wt ER both in the presence or in the absence of the coactivator GRIP1. ER activity was assayed in the four strains after incubation in raffinose medium containing 0.1 nM 17-β-estradiol. Data were collected in the same experiment but are displayed with separate *y* axes to more clearly demonstrate GRIP1 induction of ER activity within each strain. Coexpression of GRIP1 increases ER transcriptional activation in both KO and PA strains approximately 10-fold, an effect that is independent of yhp23 expression. Note that yhp23 increased ER transcriptional activation to the same extent in the absence or presence of GRIP1.

The recent determination of the structure of the ER ligand-binding domain bound to estradiol has suggested that proper folding of this region around the steroid hormone is crucial to the formation of an AF-2 domain competent for interaction with coactivators (3). Since p23 has been proposed to have chaperone-like activities (17), we hypothesized that it may play a role in the proper folding of the AF-2 domain around estradiol during the process of steroid binding. To determine whether yhp23 facilitates wt ER AF-2–coactivator interactions, we introduced the mammalian coactivator GRIP1 into yhp23 KO and PA yeast strains expressing wt ER (KO+GRIP1 and PA+GRIP1, respectively). Coexpression of GRIP1 should activate AF-2, thereby increasing wt ER transcriptional activation relative to the control strains lacking GRIP1 (22) and allowing us to compare GRIP1 induction of wt ER activity in the presence or absence of yhp23. If yhp23 were important for AF-2 interaction with GRIP1, then GRIP1-dependent ER transcriptional activation would be reduced in the KO versus the PA strain. However, as shown in Fig. 5, this is not the case. wt ER transcriptional activation in the PA and KO strains is enhanced to similar extents (approximately 10-fold) compared to the corresponding control strains lacking GRIP1 (Fig. 5). Thus, GRIP1 induction of wt ER transcriptional activation is not altered by yhp23 expression, suggesting that yhp23 is not required for the formation of a functional ER-GRIP1 complex.

ER and yhp23 colocalize within the nucleus of yeast cells. Prior genetic and biochemical studies have demonstrated that aporeceptor complex formation is conserved in yeast cells (5). Hsp82, the yeast homologue of Hsp90, has been shown to associate with hormone-free ER and GR in yeast cells. In addition, genetic studies indicate that ER and GR signaling is

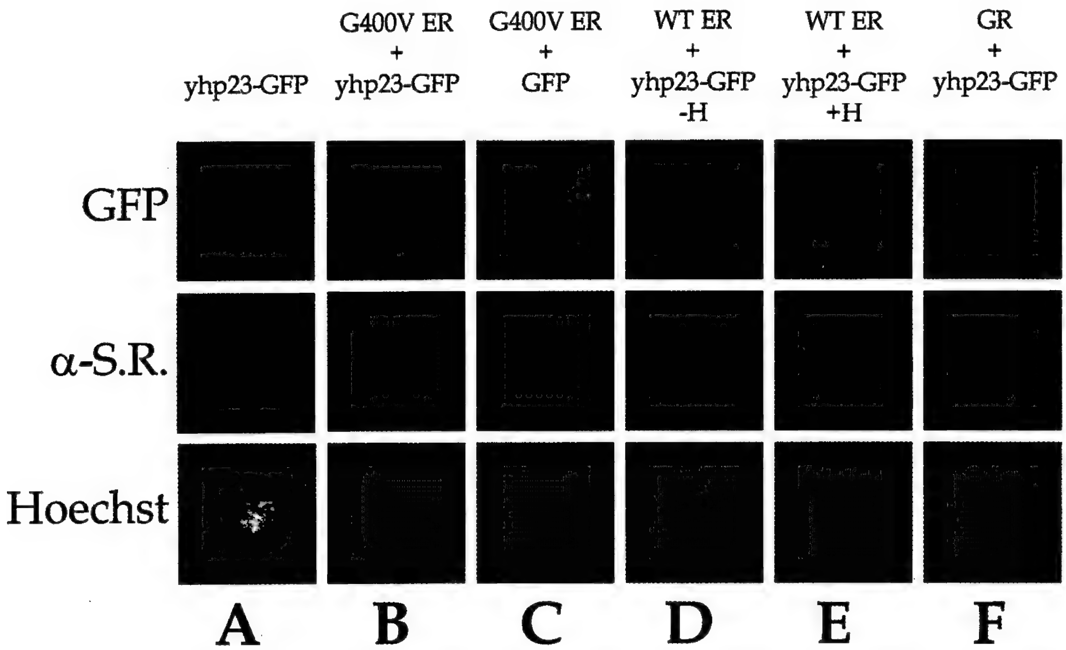

FIG. 6. ER and yhp23-GFP colocalize within the nucleus of yeast cells. To examine yhp23 subcellular localization in yeast cells, a yhp23-GFP fusion protein was constructed. Yeast strains were created that express yhp23-GFP (A) or GFP (C) either alone or in combination with G400V ER (B and C), wt ER (D and E), or GR (F). Cells were grown in galactose-raffinose-containing medium in the absence or the presence of 17-β-estradiol. Cells were fixed, permeabilized, and incubated with the appropriate receptor primary antibody, a corresponding Texas-red-conjugated secondary antibody, and the DNA in the nucleus was stained with Hoechst dye H334211. The GFP, Texas red, and Hoechst fluorescent signals were visualized by using a Zeiss Axioplan 2 fluorescence microscope. Note that yhp23-GFP is expressed throughout the cytoplasm in the absence of ER expression. In the presence of coexpressed G400V ER as well as wt ER, yhp23-GFP becomes localized to the nucleus. Incubation of the wt ER strain in 1 μM 17-β-estradiol results in the redistribution of yhp23-GFP from the nucleus to the cytoplasm, thereby reversing the ER-yhp23-GFP colocalization observed in the absence of estradiol. α-S.R., anti-steroid receptor primary antibody.

reduced in yeast strains expressing only 5% of the wt level of Hsp82 (44). Compelling genetic evidence also exists for the role of the yeast Hsp70 (31), p60 (8), Hsp40 (31), and immunophilin (14) homologues in steroid signaling in yeast cells, with the majority of these proteins having been shown to associate with GR in the absence of hormone (2, 7).

Given human p23's presence in the aporeceptor complexes of PR (28) and GR (11) and having shown that yhp23 affects ER function, we proceeded to determine whether yhp23 and ER colocalize in vivo. To determine the cellular distribution of yhp23 in yeast cells, we created a yhp23-GFP fusion protein by subcloning GFP at the carboxy terminus of the yhp23 protein. Expression of the fusion protein was confirmed by Western blotting. Importantly, the yhp23-GFP fusion protein is also able to suppress the G400V ER phenotype (not shown), proving that addition of the GFP moiety does not eliminate yhp23's ability to functionally interact with G400V ER.

We constructed several yeast strains that express yhp23-GFP either alone or in combination with G400V ER, wt ER, or wt GR. Figure 6A demonstrates that the distribution of yhp23-GFP in the absence of steroid receptor expression is largely cytoplasmic, though a small proportion of signal corresponding to the nucleus is also evident. This pattern is consistent with the expression pattern described for the human p23 protein within mammalian cells (28). Strikingly, upon coexpression of G400V ER, yhp23-GFP becomes predominantly limited to the nucleus, thus colocalizing with G400V ER (Fig. 6B). Recall that ER is a steroid receptor that resides in the nucleus in the absence of hormone. Importantly, this pattern of nuclear localization was not seen when G400V ER was coexpressed with just the GFP protein, indicating that yhp23 is responsible for the localization of the fusion protein to the nucleus (Fig. 6C). Nuclear localization of yhp23-GFP was also observed when it was coexpressed with wt ER (Fig. 6D). Thus, the ability of G400V ER to colocalize yhp23-GFP in the same manner as wt ER suggests that the G400V ER phenotype is not a result of deficient aporeceptor complex formation. Additionally, when cells coexpressing wt ER and yhp23-GFP were incubated in 17-β-estradiol, yhp23-GFP redistributed to the cytoplasm, reestablishing the pattern seen in yeast cells lacking ER expression (Fig. 6A). As a final control, yhp23-GFP coexpressed with GR, a steroid receptor that exists outside the nucleus in the steroid-free state, did not localize to the nucleus (Fig. 6F) but instead showed a cytoplasmic distribution similar to that of GR.

The colocalization of yhp23 and ER is consistent with the proposed role of yhp23 as a member of the ER aporeceptor complex. Upon coexpression with ER, aporeceptor complex formation causes yhp23, presumably through an interaction with Hsp82 (the yeast homologue of Hsp90), to become localized to the nucleus. The addition of hormone appears to result in the dissociation of the aporeceptor complex, allowing yhp23 to redistribute throughout the cell.

Complementation of yhp23 by human p23 in yeast cells. We next examined whether human p23, when ectopically expressed in yeast cells, functions like yhp23 to suppress the G400V ER phenotype. We established yeast strains that express G400V ER and an ERE-responsive reporter plasmid in the presence of HA-tagged human p23 (HA-p23) or HA-yhp23. A third strain containing the expression vector without an insert (vector) was used as a negative control. As shown in Fig. 7A, human p23 is capable of increasing hormone-dependent G400V ER transcriptional activation in yeast cells, although to a lesser degree than yhp23 (4-fold and 13-fold, respectively). The reduced G400V ER transcriptional activity is not a function of reduced human p23 expression relative to yhp23, since immunoblot analysis with an antibody directed against the HA

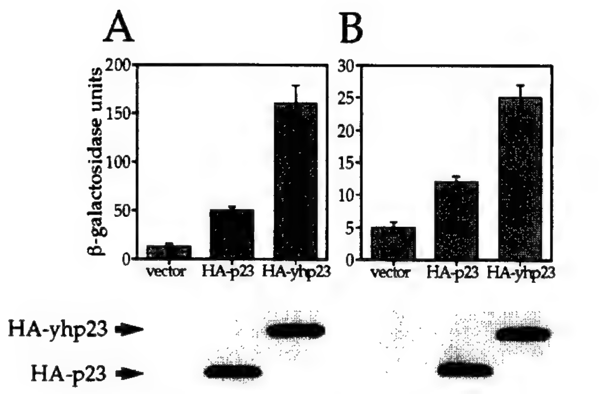

FIG. 7. Complementation of yhp23 by human p23 in yeast cells. (A) Overexpression of HA-p23 suppresses the G400V ER phenotype. An HA epitope-tagged human p23 (HA-p23) was subcloned into the yeast expression vector pRS316$_{GPD}$. The W303a yeast strain expressing G400V ER and an ERE–β-galactosidase reporter gene was transformed with expression vectors containing no insert (vector), HA-p23, or HA-yhp23. G400V ER transcriptional activity was measured by liquid β-galactosidase assay in galactose-containing medium with 1 nM 17-β-estradiol. Equal amounts of whole-cell lysates from the strain containing vector, HA-p23, and HA-yhp23 were analyzed by immunoblotting by using anti-HA antibody as described in Materials and Methods (bottom panel). (B) Human p23 partially complements the loss of yhp23 with respect to ER signaling. yhp23 KO yeast strain expressing wt ER and an ERE-responsive β-galactosidase reporter gene were transformed with expression vectors containing no insert (vector), HA-p23, or HA-yhp23. ER transcriptional activity was determined by liquid β-galactosidase assay in raffinose medium containing 0.1 nM 17-β-estradiol. Immunoblot analysis for HA-p23 and HA-yhp23 was performed as in panel A and demonstrates that HA-p23 and HA-yhp23 are expressed at similar levels (bottom panel).

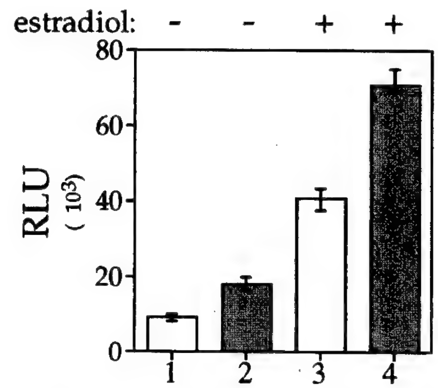

FIG. 8. Activation of ER transcriptional enhancement by p23 overexpression. ER-containing MCF-7 cells (2.5×10^5 cells/6-cm dish) were transiently transfected by using the lipid Trans-IT 100 with 5 μg of the ERE-containing luciferase reporter plasmid and 2 μg of a pCMV expression vector (open columns) or pCMV-HA-p23 expression vector (shaded columns). Cells were incubated for 24 h in the presence of 0.01 nM 17-β-estradiol or ethanol vehicle and then harvested. ER transcriptional activation was measured by using a luciferase assay, normalized to total protein concentration in each sample, and expressed as relative luminescence units (RLU). The data represent the mean of an experiment done in triplicate, which was repeated four times.

epitope present on both proteins shows equal expression levels in yeast cells (Fig. 7A, bottom panel). In addition, G400V ER expression is unaffected by yeast or human p23 coexpression (data not shown). These findings suggest that human p23 can function like yhp23 in yeast cells, albeit less potently, to increase G400V ER transcriptional activity.

We next evaluated whether human p23 could function in yeast cells to increase ER transcriptional activation in the absence of endogenous yhp23. The KO strain expressing wt ER and an ERE-responsive promoter were transformed with the empty expression vector, HA-p23, or HA-yhp23. When assayed at a 0.1 nM concentration of 17-β-estradiol, expression of human p23 increases ER transcriptional activity compared to the use of the vector only (Fig. 7B), but to a lesser extent than with yhp23 (twofold and fivefold, respectively). Again, the reduced activity of ER in the human p23-expressing strain is not a function of reduced p23 levels relative to yhp23 (Fig. 7B, bottom panel). These findings indicate that human p23 can partially complement the loss of yhp23 function in yeast cells with respect to ER signaling, thus suggesting that yhp23 and p23 are functional homologues.

Increased ER transcriptional activation by human p23 overexpression in MCF-7 cells. To establish whether p23 affects ER signal transduction in mammalian cells, we examined the ability of human p23 to increase ER-mediated transcriptional enhancement when overexpressed in cultured mammalian cells. ER-containing MCF-7 cells were transfected with the reporter plasmid ERE-thymidine kinase-luciferase and a plasmid encoding human p23. Transfected cells were treated with 0.1 nM 17-β-estradiol or ethanol vehicle for 24 h, and the transcriptional activity was quantified by measuring the luciferase activity. As shown in Fig. 8, both hormone-dependent and hormone-independent ER transcriptional activity are increased roughly twofold when p23 is overexpressed. This likely represents an underestimate of p23's importance to ER function, since these results are obtained in a cell line that contains endogenous p23 (not shown). These findings suggest that p23 is a limiting factor for ER signal transduction and therefore subsequent ER-mediated transcriptional enhancement.

DISCUSSION

Using dosage suppression analysis in yeast cells to isolate factors involved in ER signal transduction, we have identified the yeast homologue of the human p23 (yhp23) as a protein that, when overexpressed, results in a 10-fold increase in G400V ER transcriptional activation. In vivo estradiol-binding assays suggest that yhp23 overexpression increases G400V ER transcriptional activity by increasing the number of estradiol-bound receptors. The effect of yhp23 overexpression was not limited to G400V ER, as it also increases both ligand binding and transcriptional activation by wt ER. No effect of yhp23 overexpression was observed on the constitutive activity of ER_{1-115}, thereby demonstrating that yhp23 does not affect AF-1 activity per se. We therefore conclude that yhp23 is a member of the reconstituted steroid receptor signaling pathway in yeast cells, acting at the step of ligand binding by the receptor. This role is consistent with the currently proposed function of human p23 in steroid receptor-aporeceptor complex formation.

By using a yeast strain deficient in yhp23 expression (KO), analyses of wt ER signaling as a function of yhp23, wt ER, and estradiol concentrations were carried out. Our studies demonstrate that the magnitude of the effect of yhp23 on wt ER transcriptional activation is inversely proportional to the concentration of both wt ER and estradiol. Thus, at low, subsaturating concentrations of estradiol, yhp23 overexpression markedly increases wt ER transcriptional activation. In contrast, at saturating concentrations of estradiol, the effect of yhp23 overexpression on wt ER transcriptional activation is comparatively small. Furthermore, the magnitude of the effect of yhp23 on wt ER transcriptional activation is greater at low, rather than high, wt ER expression levels. Taken together, our findings indicate that the effect of yhp23 on wt ER signaling varies depending on yhp23, wt ER, and estradiol concentrations.

Subcellular localization studies with a yhp23-GFP fusion protein indicate that yhp23 is largely cytoplasmic in the ab-

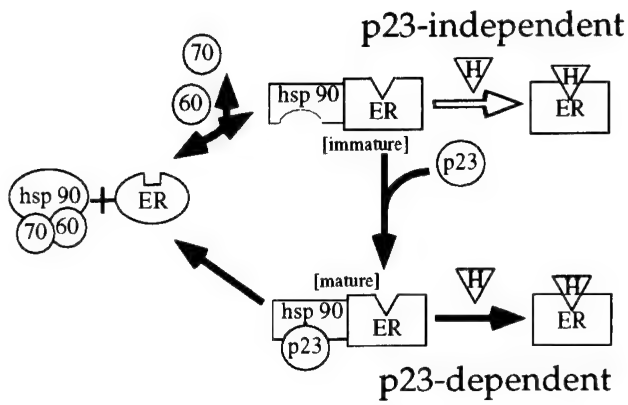

FIG. 9. A model for p23-dependent and -independent ER signal transduction. A simplified version of the current model of aporeceptor complex formation as deduced from in vitro studies is indicated by the black arrows. Hsp90 refers to the Hsp90 dimer, 70 refers to Hsp70, 60 refers to p60, p23 refers to the mammalian p23 protein, ER refers to the estrogen receptor, and H refers to hormone. The immunophilins, which do not appear to be essential to aporeceptor complex formation but are isolated with the complex in vivo have been excluded from the model for simplicity. According to the model, p60, Hsp70 (and possibly Hsp40), and a dimer of Hsp90 preassociate to form the "foldosome" complex. The foldosome binds to the steroid-binding domain of the native receptor. Hsp70 and p60 are released from the complex in a process that requires ATP and a monovalent cation, while Hsp90 and the receptor undergo conformational changes, such that the receptor assumes a conformation with high affinity for steroid. This new complex (labeled "immature" in this figure) is inherently unstable and quickly disassociates unless p23 binds to Hsp90, thereby stabilizing the aporeceptor complex (labeled "mature" in this figure). Our data, however, suggests that a second, p23-independent pathway to ER ligand binding exists in vivo, occurring when ligand binds directly to the immature aporeceptor complex (open arrow), which is favored at high ER and/or estradiol concentrations. We suggest that the G400V ER mutation renders the receptor less capable of participating in the p23-independent pathway, thereby functionally uncoupling the p23-independent and -dependent pathways.

sence of ER expression. When coexpressed with either the mutant or wt ER, yhp23 colocalizes with the receptor to the nucleus in the absence of estradiol. This colocalization is reversed upon estradiol treatment, such that yhp23 is released into the cytoplasm. From these observations, we conclude that yhp23 is part of the ER aporeceptor complex in yeast cells and that the distribution of yhp23 within the cell is dynamic and affected both by ER expression and estradiol binding.

It has been proposed that the function of p23 in steroid receptor signaling is to promote, through its interaction with Hsp90, the maturation or stabilization of the aporeceptor complex (24, 30). This model, derived largely from in vitro experiments (45, 46), proposes that the heat shock proteins Hsp90, p60, Hsp70, and possibly Hsp40 (10) form a complex termed a "foldosome" (23), within which Hsp90 exists in a conformation incompatible with p23 binding (53) (Fig. 9). The foldosome binds to the free receptor, which exists in a conformation with low affinity for the ligand. In a process that requires ATP and monovalent cations, the Hsp90 component of the foldosome and the receptor undergo conformational changes (13), such that Hsp90 is now capable of binding p23 (53), and the receptor exhibits high-affinity steroid binding. p23 binding to Hsp90 appears to stabilize this immature aporeceptor complex in vitro. In the absence of p23, the Hsp90-receptor complex is inherently unstable and rapidly dissociates (13).

The ability of yhp23 to increase ER transcriptional activation might be expected if yhp23 is limiting for the formation of mature aporeceptor complexes. Although we cannot exclude the possibility that yhp23 overexpression increases the ratio of yhp23 binding to each molecule of ER, this idea runs contrary to the current model of p23 function. Increasing the concentration of yhp23, therefore, would be expected to result in a greater number of mature ER aporeceptor complexes in the cell. As a result, the total estradiol binding will increase, which is consistent with our in vivo estradiol binding assays (Fig. 3). Thus, overexpression of yhp23, by increasing the number of mature aporeceptor complexes, will manifest itself as an increase in transcriptional activation by ER at a given hormone concentration (Fig. 2 and 4). Thus, our in vivo findings are consistent with the current model of p23 function as derived from in vitro experiments.

Our results indicating that wt ER activity is detectable in the absence of yhp23 (Fig. 4), however, suggest that in addition to the p23-dependent pathway, there must also exist a p23-independent pathway leading to estradiol binding and signaling by the ER. We propose that p23-independent activation of the ER in vivo occurs through estradiol binding directly to the immature (p23-deficient) aporeceptor complex (Fig. 9). This hypothesis is consistent with in vitro observations that in the absence of p23, the foldosome proteins Hsp90, Hsp70, and p60 are sufficient to induce the hormone-binding conformation of steroid receptors (13). We therefore propose that estradiol binding by the ER is a composite of both the p23-independent and p23-dependent pathways. The relative contribution of each pathway to ER activation is dependent upon the concentration of p23, ER, and estradiol (see below).

One prediction of our model is that yhp23 becomes less relevant to ER activation as the ratio of immature to mature aporeceptor complexes increases. The ratio of the two types of aporeceptor complexes is, in turn, a reflection of both ER and yhp23 concentrations. Increasing ER expression when p23 levels are constant results in a greater number of the immature aporeceptor complexes, which favors hormone binding through the p23-independent pathway. Conversely, increasing yhp23 levels facilitates the formation of mature aporeceptor complexes and therefore the p23-dependent pathway. This model of ER signal transduction is consistent with our in vivo findings that indicate that the magnitude of the effect of yhp23 on ER transcriptional activation is inversely proportional to the concentration of ER (Fig. 4D).

The proposed model further envisions that the concentration of estradiol also affects the relative contribution of the p23-dependent and p23-independent steroid binding pathways. As suggested by Fig. 9, free steroid can be considered to be competing with yhp23 for binding to the immature aporeceptor complex. As a result, the p23-independent pathway becomes more prominent as estradiol concentrations rise or, conversely, as yhp23 levels fall. Consistent with this notion, our data demonstrate that the magnitude of yhp23's effect on ER signaling is greatest at low subsaturating, rather than high saturating, estradiol concentrations (Fig. 4E). Thus, our model predicts that the balance among yhp23, ER, and estradiol, ultimately determines the relative contributions of the p23-dependent and p23-independent pathways to ER signal transduction.

Our model also provides insight into the observation that yhp23 overexpression induces G400V ER activity to a greater extent than that observed for wt ER (Fig. 2). We suggest that by altering the steroid-binding domain conformation, the G400V mutation largely eliminates the p23-independent pathway. G400V ER is temperature sensitive, relative to the wt ER, for estradiol binding in vitro, displaying reduced estradiol binding at 25°C but not at 4°C (54). This suggests that the G400V ER mutation destabilizes the conformation of the steroid-binding domain, such that the receptor is unable to bind steroid with high affinity at 25°C. This mutation does not inhibit G400V ER's interaction with the aporeceptor complex since G400V ER has been reported to be complexed with Hsp90 (1)

and since our subcellular localization studies suggest that G400V ER associates with yhp23 as efficiently as does wt ER. We therefore propose that the G400V mutation, by altering the structure of the steroid-binding domain, largely inhibits estradiol binding to the transient, immature aporeceptor complex, thereby diminishing estradiol binding through the p23-independent pathway. As a result, yhp23 competes more effectively with estradiol for binding to the complex, favoring the p23-dependent estradiol-binding pathway. The stability gained through yhp23 binding to the Hsp90-chaperone machinery, in turn, facilitates steroid binding by G400V ER.

It has been suggested by others that wt ER does not stably interact with Hsp90. This conclusion is based, in part, on studies with a VP16-Gal-ER_{LBD} fusion protein (1, 37). Although these constructs continue to exhibit ligand-dependent activation, association of this construct with Hsp90 could not be demonstrated. While there are a number of possible reasons for this discrepancy, we feel that the most probable explanation is a difference in the inherent characteristics of the wt and fusion proteins (1). In addition, there are several reports that support a role for Hsp90 in the process of ligand binding by ER. (i) ER has been isolated with members of the aporeceptor complex from MCF-7 cells (52) and bovine uterus (49). (ii) Hsp90 has been demonstrated to colocalize with ER in the nucleus (38). (iii) ER function has been found to be affected by mutant Hsp90 molecules (44). (iv) ER function is also affected by the Hsp90-specific inhibitor geldanamycin (51). Although these studies do not exclude the possibility of additional transcriptional repressors of wt ER, these findings, along with our results, strongly support a role for the components of the Hsp90-based chaperone complex in ER signal transduction.

Elegant genetic studies of yhp23 function in yeast cells by Bohen (2) and Fang et al. (16) demonstrate that yhp23 associates with Hsp90 and is a part of the GR aporeceptor complex in yeast cells. In contrast to our findings with ER, analysis of androgen receptor (AR) signaling in yeast cells suggests that it is largely p23 independent. This may reflect inherent differences in the mechanism of signal transduction employed by the receptors. Alternatively, we would suggest that, although the analysis of AR signaling was performed under a range of steroid concentrations, the levels of AR expression used may have favored the p23-independent pathway. It would be interesting to reevaluate AR signaling as a function of yhp23 at both low AR and testosterone concentrations, conditions that would favor the p23-dependent pathway.

The partial complementation of human p23 in yeast cells lacking yhp23 strongly suggests that yhp23 functions as the p23 homologue with respect to ER signaling (Fig. 7). Although the yeast and human p23 proteins have regions of identity, significant sequence differences between the proteins also exist (2). We speculate that this reflects species-specific differences in p23-Hsp90 association and might therefore explain the inability of human p23 to fully complement the loss of yhp23 function. It would be interesting to examine whether yeast cells expressing human Hsp90 (44) and p23 increase ER activity to the same extent as their yeast counterparts.

Finally, our studies also provide insight into the possible mechanisms by which the ER communicates with other signaling pathways. Unexpectedly, a significant increase in estradiol-independent activation of ER was observed as a result of yhp23 overexpression (Fig. 4F). This estradiol-independent activation of wt ER was also observed upon p23 overexpression in MCF-7 cells (Fig. 8). Previous studies have proposed that estradiol-independent transcriptional activation by wt ER results, in part, from ER phosphorylation through an epidermal growth factor-dependent pathway (4, 27). Thus, the most direct interpretation of our data suggests that maintenance of ER within the aporeceptor complex facilitates (but is not essential to) estradiol-independent activity, perhaps by maintaining ER in a conformation amenable to phosphorylation.

Besides ER, several other signaling molecules (including c-Src and c-Raf) are dependent upon chaperone complexes for their function (45). Yeast cells deficient in the DnaJ homologue YDJ1, for instance, display both altered steroid receptor and Src kinase activity (31). Thus, molecular chaperones link diverse signaling pathways. Additional insight into the mechanism of this cross talk comes from our subcellular localization studies that reveal a striking colocalization of ER and yhp23 within the nucleus. Estradiol treatment was shown to liberate yhp23 (and presumably other chaperone proteins) from the nucleus, allowing it to redistribute throughout the cytoplasm, where it can potentially interact with other signaling proteins. Although our colocalization studies were carried out under conditions of overexpression, we speculate that estradiol activation of ER may, through the release of chaperone components, modulate the activity of a variety of chaperone-dependent pathways. In light of p23's role in stabilizing chaperone complexes, it is likely to play a key regulatory role in any such "chaperone signaling."

In conclusion, we have provided evidence that yhp23 is a member of the ER signaling pathway and a positive regulator of ER function. We also suggest that at high ER and/or estradiol concentrations, conditions often present in the yeast system or during transient overexpression of ER in cultured mammalian cells, ER signaling occurs largely through a p23-independent pathway. Under low physiological concentrations of ER and estradiol, p23 is likely to be an important contributor to ER signaling. Our results also indicate that alterations in the level or subcellular distribution of p23 are potential mechanisms for modulating estradiol-dependent and -independent ER transcriptional activation. We are currently examining whether the p23 levels, the subcellular distribution, or the modification state fluctuates between normal and tumor cells, during cellular proliferation, during differentiation, or upon growth factor treatment. As aporeceptor complex formation is also believed to be important for ligand binding by GR, PR, and AR, p23 likely plays an important role in these pathways as well.

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Potentiation of Human Estrogen Receptor α Transcriptional Activation through Phosphorylation of Serines 104 and 106 by the Cyclin A-CDK2 Complex*

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Both estradiol binding and phosphorylation regulate transcriptional activation by the human estrogen receptor α (ER). We have previously shown that activation of the cyclin A-CDK2 complex by overexpression of cyclin A leads to enhanced ER-dependent transcriptional activation and that the cyclin A-CDK2 complex phosphorylates the ER N-terminal activation function-1 (AF-1) between residues 82 and 121. Within ER AF-1, serines 104, 106, and 118 represent potential CDK phosphorylation sites, and in this current study, we ascertain their importance in mediating cyclin A-CDK2-dependent enhancement of ER transcriptional activity. Cyclin A overexpression does not enhance transcriptional activation by an ER derivative bearing serine-to-alanine changes at residues 104, 106, and 118. Likewise, the cyclin A-CDK2 complex does not phosphorylate this triple-mutated derivative *in vitro*. Individual serine-to-alanine mutations at residues 104 and 106, but not 118, decrease ER-dependent transcriptional enhancement in response to cyclin A. The same relationship holds for ER phosphorylation by cyclin A-CDK2 *in vitro*. Finally, enhancement of ER transcriptional activation by cyclin A is evident in the absence and presence of estradiol, as well as in the presence of tamoxifen, suggesting that the effect of the cyclin A-CDK2 on ER transcriptional activation is AF-2-independent. These results indicate that the enhancement of ER transcriptional activation by the cyclin A-CDK2 complex is mediated via the AF-1 domain by phosphorylation of serines 104 and 106. We propose that these residues control ER AF-1 activity in response to signals that affect cyclin A-CDK2 function.

The estrogen receptor α (ER),[1] a transcription factor that controls the expression of a number of genes involved in cellular differentiation and proliferation in a wide variety of tissues (1–4), is regulated by ligand binding and phosphorylation. The receptor is structurally similar to other members of the nuclear receptor superfamily in that separate receptor activities such as DNA and ligand binding are localized to distinct regions of the protein (5). ER contains at least two transcriptionally active domains: constitutively active AF-1 in the N terminus of the protein and ligand-dependent AF-2 at the ER C terminus. AF-1 and AF-2 can act independently or synergize to effect transcriptional activation (6, 7). Interestingly, they are differentially affected by certain ligands such as tamoxifen, which blocks AF-2 action but activates AF-1, accounting for the mixed agonist-antagonist properties of this agent (8, 9).

Although ligand binding is considered essential for the full activation of ER, it has long been recognized that the receptor is subject to post-translational alterations, such as phosphorylation, which also regulate its activity (10). The phosphorylation of three N-terminus-located residues, serines 104, 106, and 118, which are the focus of our current studies, appears to regulate receptor-dependent transcriptional activation (11, 12). This additional level of regulation most likely serves to modulate receptor activity in a cell- and physiologically-specific manner. Indeed, it has been suggested that phosphorylation of steroid receptors may determine promoter specificity, cofactor interaction, strength and duration of receptor signaling, and ligand-independent receptor transactivation. Since ER can serve as a transcriptional repressor as well as an activator, effecting cellular proliferation in some settings and arrest or differentiation in others (13–17), this level of complexity and flexibility is not surprising.

Much work has been directed toward elucidating which circumstances induce ER phosphorylation and which receptor sites are the targets for this modification. Although a number of potential phosphorylation sites have been identified, the kinases that modify these residues are not fully established. In addition, ER phosphorylation patterns appear to be cell type-specific. Serine residues are the predominantly modified amino acids present in ER, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the N terminus within AF-1 of the receptor (12). The sequence context surrounding serines 104, 106, and 118 suggests that they may be targeted by the serine/proline-directed protein kinases, which include mitogen-activated protein kinase family members, glycogen synthase kinase-3, and the cyclin-dependent kinases (CDKs). Indeed, Ser-118 has been shown to be phosphorylated by the mitogen-activated protein kinase family member, extracellular signal-regulated kinase 1 (ERK-1), *in vitro* and to facilitate ER ligand-independent activation *in vivo* (18, 19). Recent findings also suggest that Ser-118 is phosphorylated by a kinase distinct from mitogen-activated protein kinase upon estradiol treatment, suggesting that Ser-118 is the target for multiple kinases *in vivo* (20). Serine 167 has been shown to be phosphorylated by $p90^{rsk1}$ *in vitro* and to regulate ER AF-1-dependent transcrip-

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[1] The abbreviations used are: ER, estrogen receptor; CDK, cyclin-dependent kinase; AF-1, activation function-1; wt, wild type; GST, glutathione *S*-transferase; E2, 17β-estradiol.

tional activation *in vivo* (21); interestingly, this site also lies within the consensus sequence targeted by both calmodulin-dependent protein kinase II and casein kinase II and has been reported to be phosphorylated by the latter *in vitro,* although the physiological significance of this finding remains uncharacterized (22). Three of the putative phosphorylation sites, serines 104, 106, and 118, are critical for ER-dependent transcriptional enhancement and are phosphorylated in COS-1 cells (11). In an attempt to identify the kinase(s) responsible for this alteration, we have previously shown that the cyclin A-CDK2 complex phosphorylates ER between residues 82 and 121 *in vitro* and that overexpression of cyclin A *in vivo* results in ligand-independent hyperphosphorylation of the receptor (23). Regulatory effects of cyclin-CDK complexes upon steroid/nuclear receptors have been described for three other family members. The glucocorticoid receptor is phosphorylated by two cyclin-CDK complexes, A-CDK2 and E-CDK2 (24). The progesterone receptor is phosphorylated by the cyclin A-CDK2 complex, and the retinoic acid receptor is phosphorylated by cyclin H-CDK7, leading to ligand-dependent enhancement of receptor transcriptional activation (25, 26).

To identify ER residues phosphorylated by the cyclin A-CDK2 complex, we have generated a series of phosphorylation site-specific mutant ER derivatives at serines 104, 106, and 118, the three potential CDK phosphorylation sites. We examined the effect of cyclin A overexpression on ER transcriptional activation of these serine-to-alanine mutants, individually and collectively, in cultured mammalian cells and also determined whether these sites are phosphorylated by the cyclin A-CDK2 complex *in vitro*. Our results suggest that the effect of cyclin A-CDK2 on ER transcriptional activation is mediated by phosphorylation of serines 104 and 106.

EXPERIMENTAL PROCEDURES

Plasmids and Generation of ER Phosphorylation Site Mutants—Phosphorylation site mutants were generated via a two-step polymerase chain reaction process wherein overlapping primers (a "top" strand and a "bottom" strand; Genelink, Thornwood, NY) bearing the mutation of interest were mixed and amplified. The reactions were carried out on a Perkin-Elmer GeneAmp 2400 System using Perkin-Elmer reagents and *Taq* DNA polymerase. Intermediate polymerase chain reaction products were separated from excess primer and template using the Qiagen polymerase chain reaction purification kit. B. Katzenellenbogen (University of Illinois, Urbana) kindly provided a double mutant, pCMV5-ER S104A/S106A. Triple phosphorylation site mutants in the context of pGex4T-1 (Amersham Pharmacia Biotech) and pcDNA3 (Invitrogen) were constructed by subcloning. All phosphorylation site mutants were sequenced to verify the existence of the desired base alterations and to guard against the inclusion of untoward mutations (Sequenase Version 2.0 DNA sequencing kit, U. S. Biochemical Corp.).

pcDNA3-wt ER, pcDNA3-ER S104A, pcDNA3-ER S106A, pcDNA3-ER S118A, and pcDNA3-ER S104A/S106A/S118A expression plasmids were used to produce full-length human ER derivatives, and an XETL reporter plasmid containing one consensus ERE upstream of firefly luciferase gene was used to assay for ER transcriptional activity. The pCMV-Myc-cycA plasmid expressed Myc-tagged cyclin A. A pCMV empty vector was used to equalize the total amount of DNA transfected in each experiment. pCMV-LacZ plasmid produced β-galactosidase and was used as an internal control for transfection efficiency.

Cell Culture, Transient Transfections, and ER Activity Assays—U-2 OS human osteosarcoma cells (ATCC HTB 96) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone), 50 units/ml each penicillin and streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.).

For transient transfections, U-2 OS cells were seeded into 60-mm dishes (120,000 cells/dish) in Dulbecco's modified Eagle's medium, 10% fetal bovine serum. One h before transfection, cells were re-fed with phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum and transfected with indicated plasmids via the calcium phosphate precipitation method as described elsewhere (27). Five h post-transfection, cells were washed three times with phosphate-buffered saline to remove calcium phosphate precipitates, allowed to recover overnight in phenol red-free Dulbecco's modified Eagle's medium, 10% stripped fetal bovine serum, and incubated with fresh medium containing 100 nM 17β-estradiol (E2, resuspended in 100% ethanol) or 1 μM 4-hydroxy-tamoxifen (Calbiochem-Novabiochem; resuspended in 100% ethanol) where indicated for an additional 12 h.

Transfected cells were washed twice with phosphate-buffered saline and lysed directly on the dishes in 250 μl of 1× reporter lysis buffer (Promega). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol. A Lumat LB 9507 luminometer (EG&G Berthold) was used with 1 mM D-luciferin (Analytical Luminescence Laboratory) as substrate. Luciferase assays were performed, normalized to β-galactosidase (28) activity, and expressed as relative luminescence units.

Immunoblotting—To prepare protein extracts from transfected cells, U-2 OS cells were washed twice with phosphate-buffered saline and lysed directly on the plates in 200 μl of ice-cold lysis buffer (150 mM NaCl, 50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 25 μM $ZnCl_2$ supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride)) and a phosphatase inhibitor, 1 mM sodium orthovanadate. The lysates were collected, incubated on ice for 15 min, and precleared by centrifugation (10,000 × g for 10 min at 4 °C), protein concentration in all samples was adjusted with the lysis buffer, and 200 μl of the whole cell extracts was boiled for 3 min with 50 μl of 5× SDS sample buffer. For immunoblotting, protein extracts were fractionated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membrane (Millipore), and probed with the Myc-specific mouse monoclonal antibody to detect transfected Myc-tagged cyclin A or with anti-ER mouse monoclonal or rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc. catalog #SC-040, SC-787 and SC-543, respectively). The blots were developed using horseradish peroxidase-coupled sheep anti-mouse or goat anti-rabbit antibodies and the Enhanced Chemiluminescence (ECL) substrate as per the manufacturer's instructions (Amersham Pharmacia Biotech).

Purification of ER Derivatives as GST Fusion Proteins and Generation of Cyclin A-CDK2 Complexes in Baculovirus Expression System—Human ER derivatives containing N-terminal amino acids 1 through 121, either wild type (wt) or containing single S104A, S106A, S118A or triple S104A/S106A/S118A amino acid substitutions were subcloned into the pGex4T-1 vector (Amersham Pharmacia Biotech) and expressed in *Escherichia coli* as glutathione *S*-transferase (GST) fusion proteins (GST-ER_{121}) as described (24). The most concentrated fractions (1 mg/ml) were used as substrates for the *in vitro* kinase assays.

High Five insect cells were maintained in Ex-Cell 405 insect culture media (JRH Biosciences) at 27 °C. Baculovirus vectors (10^7 plaque-forming units/ml) engineered to express human cyclin A or a hemagglutinin-tagged human CDK2 were used separately or in combination to infect cells. Cells (1×10^7 cells/100-mm dish) were infected with 0.5 ml (5×10^6 plaque-forming units) of each virus in a final volume of 2.5 ml for 3 h at 27 °C and re-fed with 10 ml of Ex-Cell medium. Two days post-infection, cells were lysed on ice for 1 h in 0.5 ml of 120 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM dithiothreitol supplemented with protease inhibitors (described above) and phosphatase inhibitors (1 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate). Lysates were cleared by centrifugation at 12,000 × g for 10 min at 4 °C, frozen on dry ice and stored at −80 °C.

In Vitro Kinase Assays—The cyclin A-CDK2 complex was immunoprecipitated from approximately 100 μg of insect cell extract for 1 h on ice with 5 μg of the monoclonal antibody 12CA5 (Roche Molecular Biochemicals) directed against the hemagglutinin epitope on CDK2. Immune complexes were immobilized on protein A/G-agarose beads (Santa Cruz Biotechnology) for 1.5 h at 4 °C, washed 3 times in 1 ml of lysis buffer (described above), once with 1 ml of lysis buffer without Nonidet P-40, and once with DK buffer (50 mM potassium phosphate, pH 7.15, 10 mM $MgCl_2$, 5 mM NaF, 4.5 mM dithiothreitol) with protease inhibitors (described above). The wild type or mutant GST-ER_{121} substrates (approximately 10 μg in 100 μl) were added to the immobilized kinase complex, the kinase reaction was initialized by adding 25 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, and [γ-^{32}P]ATP (100 μCi) in a total volume of 300 μl and allowed to proceed for 30 min at room temperature with continuous shaking. Reaction mixtures containing the immobilized receptor on glutathione beads and recombinant purified ERK-2 (New England Biolabs) were set up according to the manufacturer's instructions. The beads containing the kinase complex and the bound substrate were then washed 3 times with 1 ml of phosphate-

buffered saline to remove unincorporated radioisotope, and the labeled GST-ER_{121} derivative was released by boiling at 100 °C for 3 min in an equal volume of 2× SDS sample buffer and fractionated on 10% SDS-polyacrylamide electrophoresis gels. The gels were stained with Coomassie Blue to visualize the receptor protein and dried, and the phosphorylation of substrates was examined by autoradiography at room temperature. To quantitate the amount of ^{32}P incorporated into each ER derivative, the receptor bands were excised from the gel, immersed in scintillation fluor, and quantitated using a scintillation counter.

RESULTS

Enhancement of ER Transcriptional Activation by Cyclin A Overexpression Is Abolished in the ER Triple Mutant S104A/S106A/S118A—We have previously demonstrated that overexpression of cyclin A in mammalian cells enhances ER transcriptional activation. To determine whether the effect of cyclin A is mediated through one or more of the three potential CDK phosphorylation sites in AF-1, Ser-104, Ser-106, and Ser-118 (Fig. 1*A*), we have substituted these serines with alanines (S104A/S106A/S118A) in the context of the full-length human receptor and compared the effect of cyclin A overexpression on the transcriptional response of the wt *versus* triple mutant ER in ER-deficient U-2 OS human osteosarcoma cells. Fig. 1*B* demonstrates that overexpression of cyclin A results in a 2-fold increase of wt ER transcriptional enhancement. The ER triple mutation S104A/S106A/S118A (AAA mutant) completely abolished the receptor response to cyclin A (Fig. 1*B*, *top panel*). Importantly, the ER AAA mutant was expressed at the same level as the wt ER, and the expression of either derivative was not affected by the exogenously transfected cyclin A (Fig. 1*B*, *bottom panel*). These results suggest that the effect of cyclin A on ER transcriptional activation is not a function of alterations in expression of ER but rather is mediated, individually or collectively, through serines 104, 106, and/or 118.

Phosphorylation of ER by the Cyclin A-CDK2 in Vitro Is Abolished in the ER Triple Mutant S104A/S106A/S118A—To examine whether Ser-104, Ser-106, and Ser-118 were indeed sites for cyclin A-CDK2 phosphorylation, we have examined whether purified cyclin A-CDK2 could phosphorylate an ER derivative containing receptor amino acid residues 1 through 121 using an immune complex kinase assay. Both the wt ER and an ER containing the three amino acid substitutions S104A/S106A/S118A (AAA) were fused to GST, expressed in *E. coli*, and purified by glutathione affinity chromatography. The cyclin A-CDK2 complex was purified from baculovirus-infected insect cells by immunoprecipitation using antibody directed against an hemagglutinin epitope present on the CDK2 subunit of the complex. As shown in Fig. 2 (*top panel*), immunopurified cyclin A-CDK2 complex phosphorylates the wt GST-ER_{121} derivative, but not the AAA mutant, *in vitro*. These results suggest that the cyclin A-CDK2 complex directly phosphorylates one or more of the serine residues, 104, 106, or 118, *in vitro*.

Serines 104 and 106, but Not 118, Mediate Cyclin A-dependent Enhancement of ER Transcriptional Activation in Mammalian Cells—ER responsiveness to cyclin A overexpression as well as ER phosphorylation *in vitro* suggests three candidate target sites for the cyclin A-CDK2-mediated phosphorylation, Ser-104, Ser-106, and Ser-118, all of which lie within the serine-proline consensus motif, potentially modified by CDKs. To determine which of these serine residues are required for the cyclin A-mediated induction of ER transcriptional activation in mammalian cells, we constructed a series of full-length ER derivatives bearing individual serine-to-alanine substitutions, S104A, S106A, and S118A. These constructs were expressed in U-2 OS cells and assayed for ER-dependent transcriptional activation under conditions of cyclin A overexpression.

Fig. 3*A* demonstrates that the ER S104A and S106A mutations, but not the S118A substitution, partially suppress the effect of cyclin A on ER transcriptional activation relative to the wt ER. These differences in ER transcriptional activity are not a reflection of alterations in the level of ER protein synthesized, since all derivatives were expressed at a comparable

A

S104 leu-asn-ser-val-**ser(P)**-pro-ser-pro-leu

S106 asn-val-ser-pro-**ser(P)**-pro-leu-met-leu

S118 pro-pro-gln-leu-**ser(P)**-pro-phe-leu-gln

B

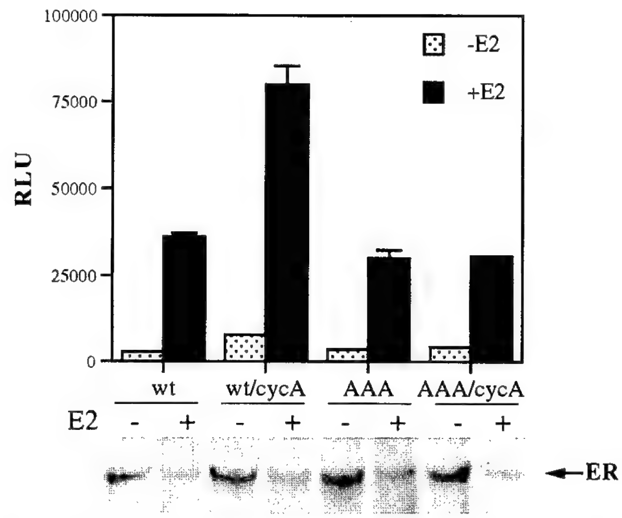

FIG. 1. **Replacement of ER N-terminal phosphorylation sites abolishes cyclin A-dependent induction of ER transcriptional enhancement in U-2 OS cells.** *A*, sequence context of Ser-Pro phosphorylation sites in ER AF-1. Shown are the amino acid residues surrounding the phosphorylation sites Ser-104, Ser-106, and S118 (in *bold*). Candidate kinases with the potential to modify these sites are CDK (consensus motif = Ser/Thr(P)-Pro-Lys/Arg), GSK-3 (consensus motif = Ser/Thr(P)-Pro-Xaa-Ser(P)), and mitogen-activated protein kinase (consensus motif = nonpolar-Xaa-Ser/Thr(P)-Pro), where Xaa is any amino acid. *B*, the ER derivative triple-mutated at Ser-104, Ser-106, and Ser-118 is not responsive to cyclin A overexpression. U-2 OS human osteosarcoma cells were transiently transfected via the calcium phosphate precipitation method with the full-length human ER (pcDNA3-ER, 1 μg/60-mm dish), either wild type (wt) or a S104A/S106A/S118A triple mutant (AAA), an XETL reporter plasmid containing a single consensus ERE upstream of a luciferase gene (2 μg/60-mm dish), a pCMV-LacZ plasmid (0.5 μg/60-mm dish), and a pCMV-Myc-cycA plasmid (cycA, 3 μg/60-mm dish) expressing Myc-tagged full-length human cyclin A, where indicated. The total amount of DNA transfected per dish was equalized with a pCMV "empty" expression vector. Receptor transcriptional activity in the absence or presence of 17β-estradiol (E2) was measured via luciferase assay 12 h after the addition of E2 to the medium, normalized to β-galactosidase activity, and expressed as relative luminescence units (*RLU*, *top panel*). The effect of cyclin A on the ER-responsive reporter did not reflect general activation of transcription, as it was dependent on ER (23), and pCMV-LacZ activity was not affected by cyclin A overexpression compared with empty vector-transfected cells. To verify equal expression of ER derivatives in the presence or absence of overexpressed cyclin A, whole cell extracts were prepared as described under "Experimental Procedures" from a set of identical dishes and the expression of the wt, and triple-mutated ER was analyzed by immunoblotting with ER-specific mouse monoclonal antibodies (Santa Cruz-787, *bottom panel*). Parental U-2 OS cells do not contain endogenous ER α based on immunoblotting as well as transcriptional activity assays (data not shown).

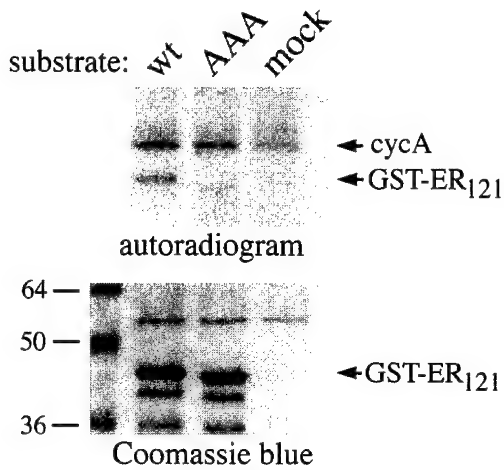

FIG. 2. **Cyclin A-CDK2 complex does not phosphorylate the ER S104A/S106A/S118A derivative *in vitro*.** GST-ER fusion proteins containing receptor amino acid residues 1 through 121 (GST-ER_{121}), either wt or containing three amino acid substitutions at receptor phosphorylation sites S104A/S106A/S118A (AAA), were expressed in *E. coli* and purified as described (24). The cyclin A-CDK2 complex was expressed in insect cells by baculovirus infection, immunopurified using anti-hemagglutinin mouse monoclonal antibodies as described under "Experimental Procedures," and added to the wt or AAA substrate for the kinase reactions. Immunopurified kinase complex without added ER substrate (*mock*) was used as negative control. The reaction products were separated on 10% SDS-polyacrylamide electrophoresis gels and stained with Coomassie Blue to visualize the substrate proteins (*bottom panel*), and autoradiography was performed (*top panel*).

level in both the presence and absence of exogenous cyclin A (Fig. 3*B*). The results from four independent experiments (Fig. 3*C)* demonstrate that S118A mutant is fully responsive to cyclin A, whereas both S104A and S106A are reduced in their response, with the average induction by cyclin A 43 and 18%, respectively. Thus, residues 104 and 106, but not 118, are responsible for the observed cyclin A-dependent enhancement of ER transcriptional activity in cultured mammalian cells. Interestingly, neither the S104A nor the S106A mutations completely eradicate cyclin A enhancement of ER activity, suggesting that both residues participate in the observed regulation. In addition, since either mutation results in more than 50% reduction of ER transcriptional enhancement, phosphorylation at these two sites is likely cooperative, such that replacement of either serine 104 or 106 with alanine partially inhibits phosphorylation of the adjacent site.

Individual Serine to Alanine Substitutions at ER Residues 104, 106, and 118 Are Differentially Phosphorylated by the Cyclin A-CDK2 Complex in Vitro—We next assessed the ability of the cyclin A-CDK2 complex to phosphorylate individual serine-to-alanine ER mutants (S104A, S106A, and S118A) *in vitro* in the context GST-ER_{121}. Fig. 4*A* illustrates that phosphorylation of each mutant, S104A, S106A, and S118A, is reduced compared with the wt ER. The *lower panel* is the Coomassie Blue-stained gel demonstrating that all receptor derivatives are expressed at comparable levels. To quantify the amount of phosphate incorporated into each ER mutant, the receptor and cyclin A bands were excised from the gel and subjected to liquid scintillation counting; ER phosphorylation was normalized to the amount of cyclin A immunoprecipitated and phosphorylated in each condition. Phosphate incorporation into the S104A derivative by cyclin A-CDK2 is decreased by more than 80%, relative to the wt ER (set as a 100%), whereas phosphorylation is virtually abolished when the S106A derivative is used as the substrate, reducing the amount of phosphorylation by more than 95% compared with the wt ER (Fig. 4*B*). To establish that the integrity of the S106A derivative is preserved, we tested it

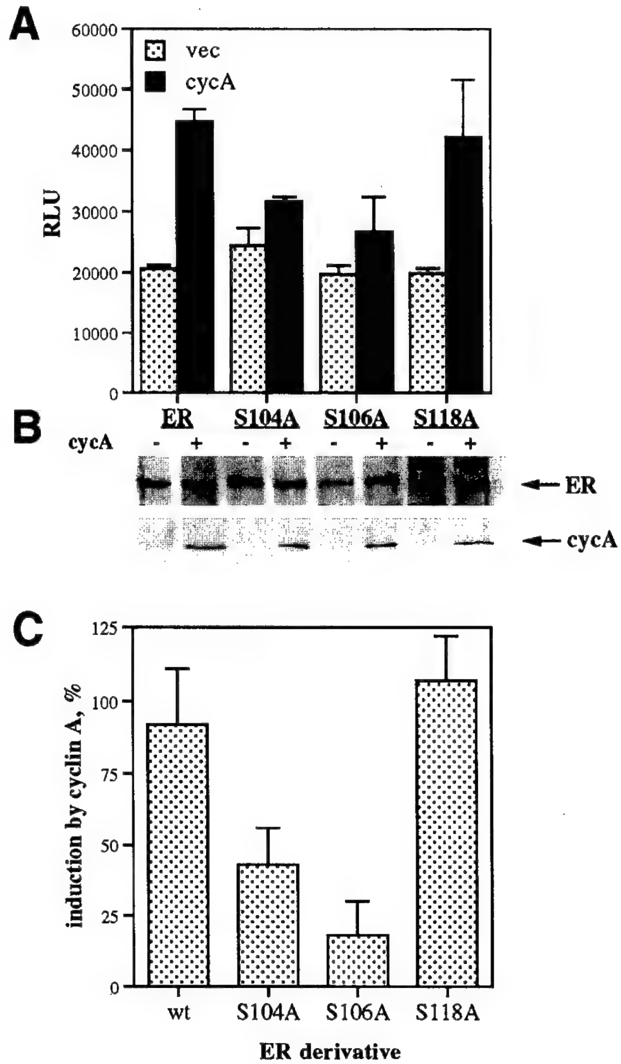

FIG. 3. **ER Ser-104 and Ser-106, but not Ser-118, are critical for cyclin A-mediated enhancement of ER transcriptional activation in U-2 OS cells.** *A*, S104A and S106A, but not the S118A mutation, abolish cyclin A-dependent induction of ER transactivation. U-2 OS cells were transfected as described in Fig. 1*B* with pcDNA3-ER (wt, S104A, S106A, or S118A, as indicated, 1 μg/60-mm dish), an XETL reporter plasmid (2 μg/60-mm dish), a pCMV-LacZ plasmid (0.5 μg/ 60-mm dish), and a pCMV-Myc-cycA plasmid (*cycA*, 3 μg/60-mm dish) or an empty pCMV vector (*vec*). ER transcriptional activation was assessed after a 12-h treatment with E2 via luciferase assay, normalized to the β-galactosidase activity, and expressed as relative luminescence units (*RLU*). *B*, ER expression level is not affected by cyclin A overexpression or point mutations at phosphorylation sites. Whole cell extracts were prepared from transfected cells as described under "Experimental Procedures," and the expression of ER derivatives and Myc-tagged transfected cyclin A was analyzed by Western blotting. *C*, differential enhancement of ER mutant transcriptional activation by overexpressed cyclin A. The average induction of transcriptional activation displayed by the wt ER and ER phosphorylation site mutants was expressed as % enhancement over the activity of each mutant in the absence of overexpressed cyclin A, which was arbitrarily set as 100%. Shown are the average and a S.E. of four independent experiments.

as a substrate for mitogen-activated protein kinase (ERK-2), which utilizes Ser-118 as a target phosphorylation site. ERK-2 readily phosphorylates S106A, suggesting that the inability of cyclin A-CDK2 to phosphorylate S106A does not result from potential changes in protein conformation induced by the mutation but rather reflects the specificity of the kinase with respect to the particular substrate site (Fig. 4*C*). The ER S118A mutation also results in a decrease in ER phosphorylation by the cyclin A-CDK2 complex, albeit to a much smaller extent

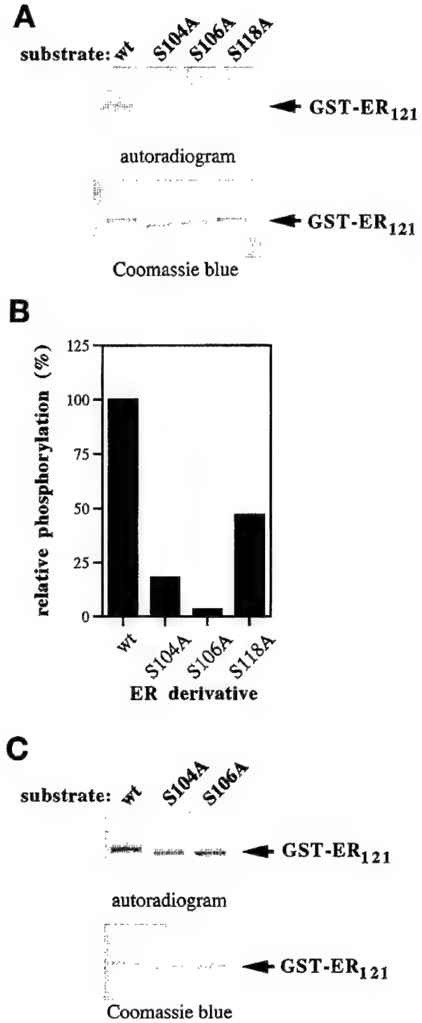

FIG. 4. **Individual mutations at ER N-terminal phosphorylation sites decrease ER phosphorylation by cyclin A-CDK2 complex *in vitro*.** GST-ER_{121} fusion proteins, either wt or containing single amino acid substitutions at receptor phosphorylation sites S104A, S106A, or S118A, were expressed in *E. coli* and purified as described above. The cyclin A-CDK2 complex was expressed and immunopurified as described in Fig. 2. Purified cyclin A-CDK2 complex (*A*) or purified recombinant ERK-2 (*C*) was added to the wt or mutant ER substrates for the kinase reactions. The reaction products were separated on 10% SDS-polyacrylamide electrophoresis gels, stained with Coomassie Blue to visualize the substrate proteins (*A* and *C*, *bottom panels*), and exposed to film (*A* and *C*, *top panels*). The GST-ER_{121} and cyclin A bands were subsequently excised from the gel and subjected to scintillation counting. ^{32}P incorporation into each ER derivative was normalized to the phosphorylation of cyclin A, immunoprecipitated in each condition. Relative efficiency of phosphorylation was calculated for each ER mutant by setting counts/min of the wt GST-ER_{121} as a 100% (*B*). Note that each serine-to-alanine substitution decreases the amount of GST-ER_{121} phosphorylation; however, S104A and S106A do so to a greater extent than S118A.

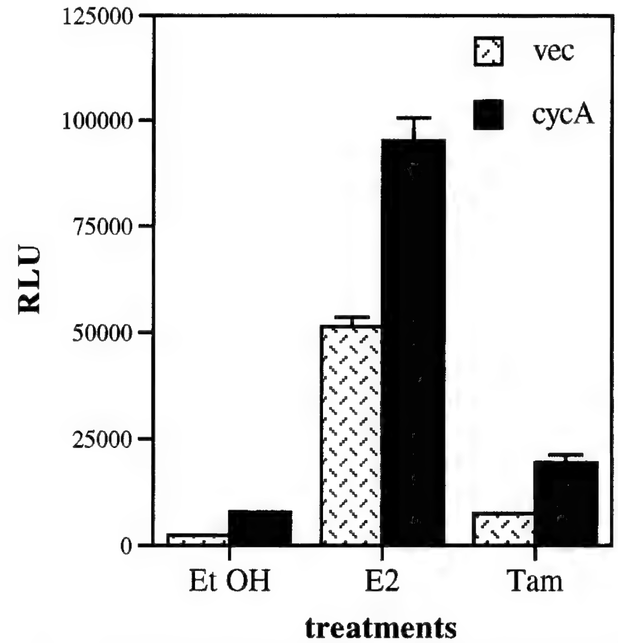

FIG. 5. **Cyclin A-mediated induction of ER transactivation is ligand-independent.** U-2 OS cells were transfected as described in Fig. 1, and ER transcriptional activation in the absence of ligand (*Et OH*), in the presence of 100 nM 17β-estradiol (*E2*), and in the presence of 1 μM 4-hydroxytamoxifen (*Tam*) was assessed via a luciferase assay, normalized to β-galactosidase activity, and expressed as relative luminescence units (*RLU*). The experiment was performed in duplicate, two times, with similar results. Note that 2–3-fold induction of ER transcriptional activation by cyclin A occurs in each of the three conditions used.

than the S104A and S106A substitutions. Although the phosphorylation of all three mutant receptor derivatives by the cyclin A-CDK2 complex *in vitro* is reduced (rank order of ER phosphorylation by cyclin A-CDK2 *in vitro*: S106A < S104A < S118A < wt), S106A and S104A substitutions most profoundly affect phosphorylation by the cyclin A-CDK2 complex. Although Ser-118 appears to contribute to ER phosphorylation by the cyclin A-CDK2 complex *in vitro*, this may result from the artificial exposure of the Ser-118 site in the truncated GST-ER_{121} fusion protein. In contrast, in the context the full-length receptor expressed in mammalian cells, Ser-118 may not be accessible to the cyclin A-CDK2 complex or may be already phosphorylated by a different kinase (such as mitogen-activated protein kinase). Combined, our results argue that the ER residues Ser-104 and Ser-106 are *bona fide* cyclin A-CDK2 targets, which is supported by our transcriptional activity assays in mammalian cells.

Cyclin A-mediated Enhancement of ER Transcriptional Activation Is AF-2-independent—Cyclin A overexpression enhances the transcriptional activity of the ER in cultured mammalian cells both in the presence and in the absence of estradiol (Fig. 1*B*). Thus, the effect of cyclin A overexpression and the activation of the ER by the cyclin A-CDK2 complex appear to be independent of ligand binding, suggesting the involvement of AF-1 but not AF-2. To further evaluate the importance of AF-2 for the enhanced ER-dependent transcriptional activation in response to cyclin A overexpression, we used a pharmacological approach and employed the ligand tamoxifen, a mixed agonist/antagonist currently used in the treatment of ER-positive breast cancers. Tamoxifen prevents the productive interaction of the ER with co-activator protein(s) necessary for transcriptional activation via AF-2, thus allowing for the assessment of changes in AF-1 activity as a function of cyclin A concentration (8, 29). U-2 OS cells were transiently transfected with the ER as well as the reporter constructs described above and treated with the ethanol vehicle, estradiol or 4-hydroxytamoxifen. For each treatment, ER transcriptional enhancement was assayed in the absence and presence of cyclin A overexpression. Consistent with our previous findings, a 2-fold increase in ER-dependent transcription was observed upon cyclin A overexpression in the absence or presence of estradiol (Fig. 5). Importantly, the magnitude of induction of ER-dependent transcriptional activation by cyclin A in response to tamoxifen treatment is comparable to that observed with estradiol (Fig. 5). Thus, the recruitment of co-activator proteins to AF-2 is dispensable for the cyclin A-mediated enhancement of ER ac-

tivity, and ER AF-1 is sufficient to confer the receptor responsiveness to cyclin A.

DISCUSSION

We have identified serines 104 and 106 of the human ER as the likely targets of cyclin A-CDK2-dependent phosphorylation. A triple serine-to-alanine mutation at residues 104, 106, and 118 abolishes both the cyclin A-CDK2-dependent increase of ER transcriptional activation in U-2 OS cells and ER phosphorylation by the cyclin A-CDK2 complex *in vitro*. Individual S104A and S106A mutations reduce the cyclin A-CDK2-dependent enhancement of ER-dependent transcriptional activation. In contrast, the S118A mutant responds like wt ER to cyclin A overexpression in mammalian cells. Similarly, phosphorylation of an ER N-terminal derivative by the cyclin A-CDK2 complex *in vitro* is significantly reduced in the S104A and S106A mutants, relative to the wt ER. Although the ER S118A mutant also exhibits decreased phosphorylation by the cyclin A-CDK2 *in vitro*, this reduction is much smaller than that exhibited by either the S104A or the S106A derivatives; in addition, this site may be artificially exposed to the purified kinase in the context of the GST-ER_{121} fusion protein. These *in vitro* findings are consistent with our results in mammalian cells, where the cyclin A-CDK2-dependent enhancement of ER transcriptional activation is reduced in ER derivatives bearing serine-to-alanine mutations at 104 and 106 but not 118. These data suggest that Ser-118 is a poor target for cyclin A-CDK2 phosphorylation *in vitro* and *in vivo*. The inability of Ser-118 to serve as a substrate for cyclin A-CDK2 is also in agreement with previous reports proposing that Ser-118 is a substrate for epidermal growth factor-activated mitogen-activated protein kinase in the absence of estradiol as well as a target for another as yet unidentified kinase(s) in the presence of estradiol (18–20). Together, these results suggest that ER is a substrate for the cyclin A-CDK2 complex, with the predominant sites of phosphorylation being Ser-104 and Ser-106. Given the close proximity of Ser-104 and Ser-106, cooperativity between the sites such that the same kinase complex modifies them and phosphorylation of one site promotes phosphorylation of the other appears likely.

It is noteworthy that the ER sites phosphorylated by the cyclin A-CDK2 complex, Ser-104 and Ser-106, reside within sequence contexts that are noncanonical CDK phosphorylation targets (Fig. 1*A*), as determined by a systematic evaluation of a panel of substrates phosphorylated *in vitro* by cyclin A-CDK2 (30). It is likely that multiple factors confer specificity and efficiency to cyclin A-CDK2-mediated phosphorylation of a given target site. For example, a noncanonical site might fold in such a way that the target is presented to the kinase in a favorable manner. Furthermore, recent findings by Schulman *et al.* (31) show that a conserved hydrophobic patch on the surface of cyclin A is involved in substrate recognition through a R*X*L motif on the substrate and that this binding is important for phosphorylation of a subset of proteins by cyclin A-CDK2 (31). Interestingly, human ER-α contains three R*X*L motifs; one such motif is located in the N terminus (amino acids 37–39), whereas two others reside in the C-terminal ligand binding domain, at residues 352–354 and 477–479, respectively. In each case, these motifs are preserved among ERs from distinct species, including human, rat, mouse, sheep, pig, and chicken, suggesting a conservation of function. It is conceivable that one or more of these motifs serve as potential docking sites for the cyclin A-CDK2 complex and facilitate ER phosphorylation at Ser-104/Ser-106 by increasing the local concentration of the substrate.

The enhancement of ER transcriptional activation by cyclin A overexpression occurs not only in the absence and presence of estradiol but is also observed when the receptor is activated by tamoxifen. Since tamoxifen induces a receptor conformation that is incompatible with coactivator binding to AF-2 (8, 29), these results suggest that cyclin A-CDK2 enhances ER transcriptional activity through AF-1 and not AF-2.

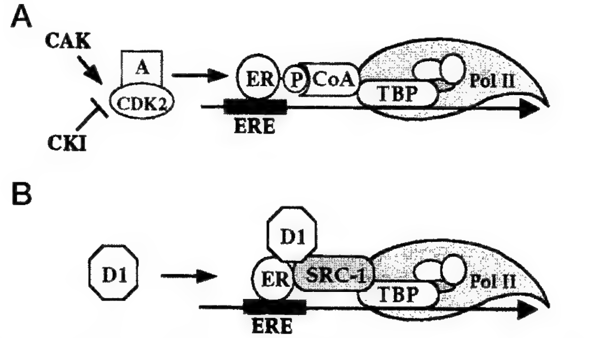

FIG. 6. **Potential mechanisms underlying ER transcriptional activation by cyclin A and cyclin D1.** *A*, a model for cyclin A-CDK2-dependent regulation of ER transcriptional activation. According to this scheme, increased expression of cyclin A leads to enhanced formation of active cyclin A-CDK2, which phosphorylates (*P*) ER at Ser-104 and Ser-106, thereby promoting the interaction between ER AF-1 and a putative co-activator (*CoA*) necessary for ER-dependent transcriptional activation. Alternatively, ER phosphorylation may dissociate a putative inhibitor (not shown). The model further envisions that increasing the concentration of positive regulators of the cyclin A-CDK2 complex, including CDK-activating complex (*CAK*) or negative regulators, for example CDK inhibitors (*CKI*) such as $p27^{KIP}$ will increase and decrease, respectively, the activity of cyclin A-CDK2, resulting in differential regulation of ER transcriptional activation. *B*, CDK-independent activation of ER by cyclin D1. Increased expression of cyclin D1 promotes the association of SRC-1 with the ER AF-2 and forms an ER-SRC-1-cyclin D1 ternary complex, thereby leading to enhanced ER transcriptional activation. *PolII*, polymerase II; *TBP*, TATA-binding protein.

We propose that ER phosphorylation at Ser-104/Ser-106 by the cyclin A-CDK2 complex provides sites that either recruit or prevent additional proteins from binding to ER AF-1. Although the p160 class of coactivators has recently been shown to interact with ER AF-1 and increase ER AF-1-dependent transcriptional activation, this effect is not dependent upon receptor phosphorylation at Ser-104, Ser-106, or Ser-118 (32). We further hypothesize that alterations in the level or activity of the cyclin A-CDK2 complex modulates ER activity by increasing or decreasing receptor phosphorylation, which in turn, affects the interaction of ER with accessory proteins involved in transcriptional regulation. This mechanism of cyclin A-CDK2 regulation of ER transcriptional activity through direct receptor phosphorylation and co-factor binding differs from that of cyclin D1-mediated enhancement of ER transcriptional activity (Fig. 6). The effect of cyclin A on ER transcriptional activation requires the kinase activity of the CDK2, whereas the effect of cyclin D1 on ER is CDK-independent (33). In addition, the enhancement of ER transactivation by cyclin A-CDK2 is achieved through phosphorylation of ER AF-1, whereas cyclin the effect of D1 on ER transcriptional activity is dependent on AF-2 and does not involve ER phosphorylation (33). Recently, it has been suggested that cyclin D1 increases ER activity by acting as a bridge between AF-2 and the coactivator, SRC-1 (34). Thus, the mechanism of cyclin D1 enhancement of ER transcriptional activity appears to be through coactivator recruitment to AF-2. Although the means whereby cyclin D1 and cyclin A augment ER transcriptional activation differs, the result is the same; that is, an increase in ER transcriptional activation either through direct coactivator recruitment to AF-2 in the case of cyclin D1, or indirectly through AF-1 phosphorylation by cyclin A-CDK2 and subsequent cofactor interaction (Fig. 6). In view of increasing clinical data linking CDK

dysregulation to a variety of human cancers, notably breast cancer (35–39), we believe that the subversion of either the cyclin D1 or the cyclin A-CDK2 pathway might account for a subpopulation of breast hyperplasias and/or tumors.

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Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins

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The hormone-activated glucocorticoid receptor (GR), through its N- and C-terminal transcriptional activation functions AF-1 and AF-2, controls the transcription of target genes presumably through interaction(s) with transcriptional regulatory factors. Utilizing a modified yeast two-hybrid approach, we have identified the tumor susceptibility gene 101 (TSG101) and the vitamin D receptor-interacting protein 150 (DRIP150) as proteins that interact specifically with a functional GR AF-1 surface. In yeast and mammalian cells, TSG101 represses whereas DRIP150 enhances GR AF-1-mediated transactivation. Thus, GR AF-1 is capable of recruiting both positive and negative regulatory factors that differentially regulate GR transcriptional enhancement. In addition, we show that another member of the DRIP complex, DRIP205, interacts with the GR ligand binding domain in a hormone-dependent manner and facilitates GR transactivation in concert with DRIP150. These results suggest that DRIP150 and DRIP205 functionally link GR AF-1 and AF-2, and represent important mediators of GR transcriptional enhancement.

Keywords: DRIP coactivator complex/glucocorticoid receptor/transactivation/TSG101

Introduction

Steroid hormones regulate multiple metabolic and developmental processes through a family of intracellular receptor proteins, termed nuclear receptors (NRs) (Tsai and O'Malley, 1994). The glucocorticoid receptor (GR), the prototype for this family of transcriptional regulatory proteins, contains an N-terminal transcriptional regulatory domain, a central Zn^{2+} finger DNA binding domain and a C-terminal region responsible for hormone binding (Yamamoto, 1985; Tsai and O'Malley, 1994). Ligand binding releases GR from the inhibitory effects of the Hsp90-based molecular chaperone complex, allowing the receptor to bind to glucocorticoid response elements (GREs), and either activate or repress transcription of specific target genes in a hormone-dependent manner (Yamamoto, 1985). GR's stimulatory or inhibitory influences on gene expression are determined by the cell context, nature of the response element and composition of interacting sequence-specific transcription factors (Miner and Yamamoto, 1992; Lefstin *et al.*, 1994; Starr *et al.*, 1996).

It has been suggested that transcriptional activators such as GR use their transcriptional activation domains as surfaces to recruit chromatin remodeling factors, and to interact with general transcription factors (GTFs) or adaptor proteins that serve to link enhancer-bound transcription factors to the GTFs, thereby initiating transcription (Chen *et al.*, 1994). These adaptor proteins are often termed coactivators. Several lines of evidence support this model of transcriptional activation by GR. At least two regions of GR possess intrinsic transcriptional activation functions (AFs). AF-2, which maps to the C-terminus, is glucocorticoid dependent, with ligand binding promoting the formation of a surface that permits protein–protein contacts between AF-2 and additional transcriptional regulatory factors. In contrast, AF-1, located at the GR N-terminus, is glucocorticoid independent or constitutive, and cofactors that associate with the GR AF-1 remain largely undefined (Godowski *et al.*, 1987; Hollenberg and Evans, 1988).

Regions within the GR AF-1 important for transcriptional activation have been identified through deletion analysis and by examining the activity of fusion proteins in yeast and mammalian cells as well as in cell-free systems. GR AF-1 has been shown to activate minimal promoter constructs in a cell-free transcription system, suggesting a direct interaction with factors involved in transcription initiation (Freedman *et al.*, 1989; Bagchi *et al.*, 1990; Elliston *et al.*, 1990; Klein-Hitpass *et al.*, 1990; Tsai *et al.*, 1990). Consistent with this idea, *in vitro* squelching assays and protein–protein interaction studies have suggested contacts between GR AF-1 and components of the RNA polymerase II transcriptional apparatus (McEwan *et al.*, 1993). Specifically, GR AF-1 interacts with the TFIID complex and the TATA binding protein (TBP) *in vitro* (Ford *et al.*, 1997). Finally, GR has been shown to enhance the formation of stable preinitiation complexes at target promoters *in vitro* (Elliston *et al.*, 1990; Tsai *et al.*, 1990). These findings support a model whereby GR AF-1 provides a surface that interacts with GTFs and possibly with as yet unidentified adaptor proteins.

A defined activation surface within GR AF-1, consisting of a 40-amino-acid 'core' between residues 208 and 247 (using the rat GR numbering scheme), has been delineated (Giguere *et al.*, 1986; Godowski *et al.*, 1988; Hollenberg and Evans, 1988; Dahlman-Wright *et al.*, 1994). This region functions as an activation domain irrespective of

its position in hybrid proteins (Hollenberg and Evans, 1988), consistent with the notion that this domain comprises an independent structural entity. Furthermore, Iñiguez-Lluhí *et al.* (1997) have identified a GR variant, termed 30IIB, that harbors three point mutations in AF-1 (E219K, F220L and W234R), which collectively abolish receptor transcriptional activation, but not transcriptional repression. Thus, the concept of a surface formed by AF-1 that recruits GTFs or novel adaptor proteins is an attractive model to define GR transcriptional activation.

Using a modified version of the yeast two-hybrid approach designed to isolate proteins that associate with transcriptional activators, we have identified proteins that associate with a functional GR AF-1. We examined the specificity of these interactions and characterized the functional effects of them on GR transcriptional enhancement in yeast and mammalian cells.

Results

A modified yeast two-hybrid approach to identify proteins that interact with GR AF-1

To identify proteins that interact with GR AF-1, we used a modified yeast two-hybrid system that allows for the selection of proteins that associate with transcriptional activators (see Materials and methods for details of the system) (Du *et al.*, 1996).

To focus our search on proteins that associate with GR in a manner dependent on the integrity of AF-1, we incorporated an additional tier of screening using the transcriptionally defective GR 30IIB mutant. Recall that this GR variant contains three point mutations in AF-1 (E219K, F220L and W234R) that selectively reduce receptor transcriptional activation by disrupting a distinct activation 'surface' while leaving the remaining architecture of the receptor intact. We observed a 95% reduction in GR AF-1-dependent transcriptional activation for 30IIB relative to wild-type (wt) GR (Figure 1A, top). Immunoblot analysis demonstrates that this decrease in GR transcriptional activation by 30IIB is not due to differences in receptor expression levels between the wt and the mutant receptor derivative (Figure 1A, bottom). Proteins that associate with the wtGR AF-1 were subsequently screened against the 30IIB mutant to identify cofactors that interact with a functional AF-1 activation surface. Out of 1.5×10^5 library transformants, 54 clones were isolated that interact specifically with the GR N-terminal activation domain, AF-1. From the 54 isolates that associated with GR, 20 displayed a reduced affinity for the 30IIB mutant (Figure 1B).

We sequenced the 20 partial cDNA clones that displayed

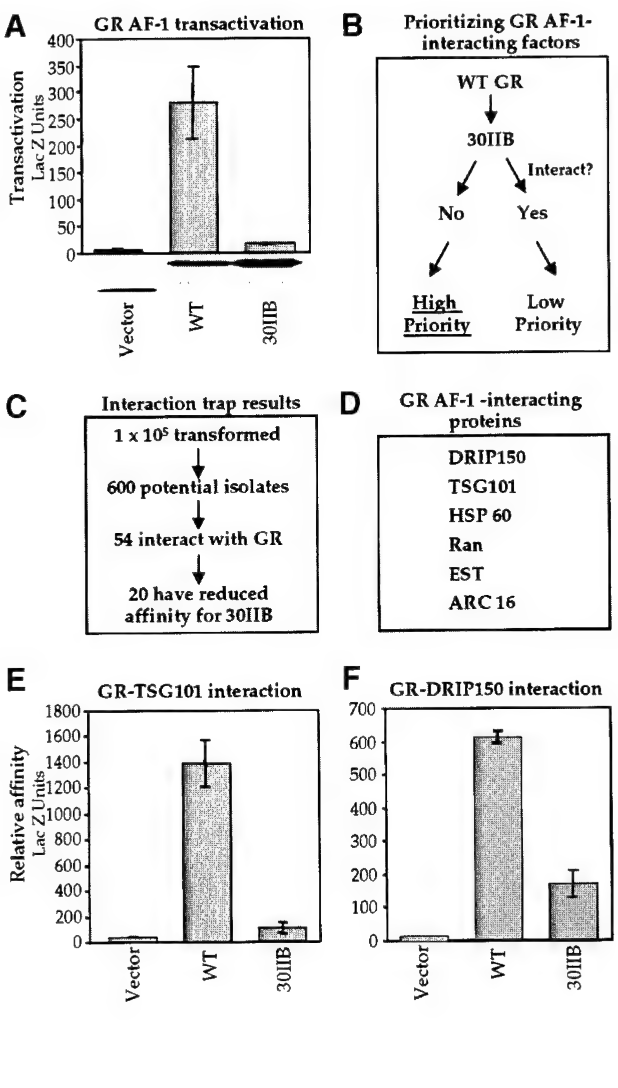

Fig. 1. Results of the modified yeast two-hybrid screen for GR AF-1 interacting factors. (**A**) Transcriptional activity of GR AF-1 derivatives. The GR wt and 30IIB mutants, in the context of the N-terminal residues 107–237, fused to the LexA DNA binding domain, were transformed into yeast along with a Lex-operator-linked β-galactosidase reporter gene. Cells were grown in selective media for 12 h at 30°C and β-galactosidase activity measured. Data represent the average of four independent clones and the standard deviation is shown. Immunoblot of extracts from strains expressing the Lex DNA binding domain (vector) or GR–LexA fusions (wt or 30IIB) were performed using a LexA-specific polyclonal antiserum. (**B**) Prioritizing GR AF-1-interacting proteins. Factors that interacted with $wtGR_{107-237}$ were screened against the GR transactivation-deficient mutant 30IIB. Isolates displaying reduced affinity for the functional activation domain as defined by the 30IIB mutant were given the highest priority. Proteins that interact with both wt and 30IIB were assigned the lowest priority. To ensure that these proteins interact specifically with GR and not with portions of the fusion protein encoded by sequences present in the vector, such as the nuclear localization signal, these clones were also screened against the activation domain construct lacking GR and showed no association with vector-encoded protein segments (not shown). The $GR_{107-237}$ bait does not associate with the LexA DNA binding domain (not shown). (**C**) Results of the interaction trap. Of the 1.5×10^5 individual HeLa cell library colonies examined for their ability to interact with GR AF-1, ~600 potential clones were selected as potential interactors ($Leu2^+/LacZ^+$); 54 associated with GR, while the remaining clones were self-activating false positives. Twenty of the 54 clones required a competent activation surface for their interaction, showing a reduced affinity for 30IIB relative to wtGR. (**D**) The 20 GR AF-1 interacting clones were partially sequenced and these sequences were used to search databases for homologies to known proteins. TSG101 and the DRIP150, along with HSP60, Ran1, ARC16 and five proteins of unknown identity represented in the EST database, interacted with wtGR, but not with 30IIB. (**E**) and (**F**) Quantitative analysis of TSG101 and DRIP150 interaction with GR AF-1 derivatives. $TSG101_{183-381}$ and $DRIP150_{1360-1454}$ expressed as fusion proteins to the LexA DNA binding domain were analyzed for their ability to interact with the vector, wtGR and 30IIB. The strength of interaction is determined by quantitative liquid β-galactosidase assays after a 12 h incubation in galactose-containing media at 30°C for TSG101 and 25°C for DRIP150, respectively. β-galactosidase activity shown represents the average from four independent colonies and the standard deviation.

reduced affinity for the GR 30IIB mutant and subjected them to a database search using the BLAST program (Figure 1C). Six of the clones were identified as HSP60, five were identical to Ran1, two matched ARC16, and five were represented in the expressed sequence tag (EST) database. Ran1, a GTPase involved in nuclear import and export, has been shown previously to affect GR nuclear localization (Carey *et al.*, 1996). ARC16, a component of the ARP2/3 complex, is involved in nucleation of actin polymerization (Welch *et al.*, 1997), while HSP60 is a chaperonin with homology to GroEL (Venner *et al.*, 1990). In addition, we identified two clones potentially involved in transcriptional regulation, which is the focus of this study. The first is the tumor susceptibility gene 101 (TSG101), which interacts strongly with the wtGR but fails to interact with 30IIB (Figure 1E). TSG101 was initially identified in a screen designed to identify tumor suppressor proteins (Li and Cohen, 1996). Inactivation of TSG101 allows naive NIH 3T3 cells to grow in soft agar and form metastatic tumors in nude mice (Li and Cohen, 1996). The predicted TSG101 protein structure is suggestive of a transcription factor (Li and Cohen, 1996). The C-terminal region contains a coiled-coil domain, the central region of the protein contains a proline-rich segment reminiscent of a transcriptional activation domain, and the N-terminus contains a catalytically inactive ubiquitin-conjugating (UBC) domain. The second protein corresponds to vitamin D receptor-interacting protein 150 (DRIP150) and displays a reduced affinity for 30IIB by 68% relative to the wtGR (Figure 1F). DRIP150 is also identical to EXLM1 (Yoshikawa *et al.*, 1998), a gene product identified in a screen for factors that fail to undergo X-chromosome inactivation. DRIP150 is a component of several multiprotein complexes. (i) DRIP150 is part of the DRIP–TRAP complex, which binds in a ligand-dependent manner to the AF-2 regions of the vitamin D receptor (VDR) and thyroid hormone receptor (TR) *in vitro*, and is required for transcriptional activation by VDR and TR *in vitro* and *in vivo* (Fondell *et al.*, 1996; Rachez *et al.*, 1998). DRIP150 does not contact AF-2 of VDR directly, but rather is brought to the VDR AF-2 by another member of the complex, DRIP205 (Rachez *et al.*, 1999). (ii) DRIP150 is also a component of the NAT complex involved in transcriptional repression, and has been termed hRGR1 due to its homology to yeast RGR1, a component of the RNA polymerase holoenzyme mediator complex (Sun *et al.*, 1998). (iii) The DRIP complex is essentially identical to the activator recruited cofactor (ARC) complex, which binds to and is required for transactivation by other transcription factors, such as SREBP-1a, NF-κB p65 and VP16 (Näär *et al.*, 1998, 1999). (iv) DRIP150 is also a member of the smaller CRSP complex (CRSP150), which is required for Sp-1 activation in a purified transcription system (Ryu *et al.*, 1999). DRIP150 is also part of the SMCC transcriptional regulatory complex (Gu *et al.*, 1999). Thus, DRIP150/EXLM1/hRGR1/CRSP150 is a gene product that escapes X-chromosome inactivation and is found in multiprotein complexes involved in transcriptional regulation.

Specificity of TSG101 and DRIP150 interactions

To analyze the specificity of the TSG101 and DRIP150 interactions, we examined their ability to associate with a panel of transcriptional regulatory proteins in the modified yeast two-hybrid assay (Figure 2). The activation domains of the cyclic AMP response element binding protein (CREB), Sp-1 (Sp-1A and Sp-1B), VP16, the AF-1 regions of the androgen receptor (AR) and TR, the steroid receptor coactivator-1 (SRC-1) and the TBP associated factor 130 (TAF 130) were utilized as baits. As shown in Figure 2, TSG101 associates with the GR, AR and TR AF-1 regions, whereas DRIP150 interacts exclusively with GR, indicating that these proteins are specific for the AF-1 domains of NRs.

GR AF-1 interacts with the coiled-coil domain of TSG101

In an attempt to localize the region of TSG101 that interacts with GR, we created a series of TSG101 C-terminal truncations. TSG101 derivatives containing amino acids 1–82, 1–121, 1–206, 1–240, 1–339 and 1–381, the full-length TSG101, were expressed as fusion proteins with LexA and their ability to interact with GR AF-1 in the modified yeast two-hybrid assay analyzed. The original TSG101 clone identified in the yeast two-hybrid screen encompasses amino acids 183–381. GR associates with the full-length $TSG101_{1-381}$ and $TSG101_{1-339}$, but not with any of the smaller TSG101 derivatives (Figure 3A). In the absence of GR AF-1 expression, on glucose plates, neither full-length TSG101 nor any of the C-terminal truncations activate transcription when bound to DNA. Immunoblot analysis using antibodies against LexA indicates that all of the TSG101 derivatives are expressed at similar levels, with the exception of $TSG101_{1-339}$ which is expressed at an ~4-fold lower level (Figure 3B), suggesting that the association between GR and $TSG101_{1-339}$ may be greater than represented in this assay. These results indicate that the region of TSG101 that interacts with GR AF-1 localizes to amino acids 241–339, a region that overlaps with the coiled-coil domain of TSG101 (Li and Cohen, 1996; Koonin and Abagyan, 1997).

DRIP150 interacts with a functional GR AF-1 in vitro

The ability of DRIP150 to interact with GR AF-1 was also tested *in vitro* using wtGR and 30IIB expressed as glutathione *S*-transferase (GST) fusion proteins. In this

Regulatory Factors	$TSG101_{183-381}$	$DRIP150_{1360-1454}$
$GR_{107-237}$	+	+
AR_{18-500}	+	-
$CREB\text{-}N_{3-296}$	-	-
$TAF\ 130_{270-700}$	-	-
$SP1\ A_{83-262}$	-	-
$SP1\ B_{263-542}$	-	-
$SRC\text{-}1_{374-800}$	-	-
$T_3R\beta 2_{1-159}$	+	-
$VP16_{412-456}$	-	-

Fig. 2. Specificity of GR–TSG101 and GR–DRIP150 interactions. Interaction of GR with $TSG101_{183-381}$ or $DRIP150_{1360-1454}$ and with other transcriptional regulatory proteins was analyzed using the modified yeast two-hybrid assay. The strength of interaction is determined by qualitative plate β-galactosidase assays after an 18 h incubation on galactose–X-gal plates at 30°C. Strong interactions (+) represent dark blue colonies and (–) represents no interaction above background 'vector only' control (white colony). TSG101 interacted with the AF-1 domains of GR, androgen receptor (AR) and thyroid receptor (TR), whereas DRIP150 associates with GR AF-1 exclusively.

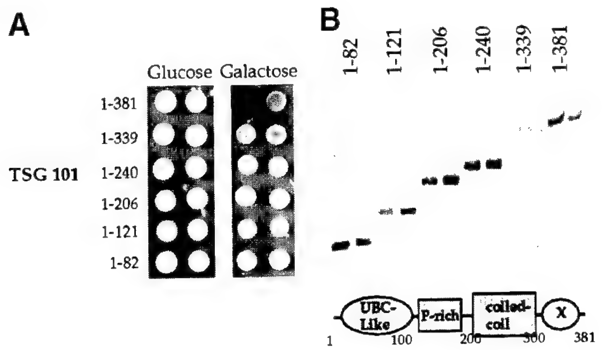

Fig. 3. GR interacts with the TSG101 coiled-coil domain. (**A**) A series of C-terminal truncations of TSG101 were fused to the LexA DNA binding domain and analyzed for their ability to interact with $GR_{107-237}$ in the yeast two-hybrid system. Expression of the β-galactosidase reporter gene was compared on glucose–X-gal versus galactose–X-gal plates. The interaction is observed when GR is expressed in the presence of galactose, but not glucose, demonstrating that the transcriptional activation is dependent on GR–TSG101 interactions. (**B**) Extracts in duplicate from strains expressing the indicated LexA DNA binding domain–TSG101 fusion proteins were probed with a LexA-specific antibody. A schematic representation of the TSG101 protein is based on structural prediction and includes an N-terminal catalytically inactive ubiquitin-conjugating (UBC-like) enzyme domain, a central proline-rich segment (P-rich) reminiscent of a transcriptional activation domain as well as a C-terminal coiled-coil domain.

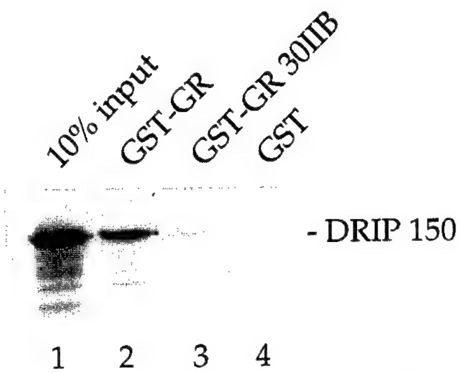

Fig. 4. DRIP150 interacts with GR AF-1 *in vitro*. Interaction of DRIP150 with the GR AF-1 'surface' *in vitro*. To examine GR–DRIP150 interactions *in vitro*, a full-length DRIP150 cDNA was translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–$GR_{107-318}$ (lane 2), GST–$GR30IIB_{107-318}$ (lane 3) or GST (lane 4). Bound DRIP150 was resolved by SDS–PAGE and visualized by autoradiography.

GST pull-down assay, *in vitro*-translated full-length DRIP150 bound GST–wtGR, but did not interact efficiently with GST–30IIB or GST (Figure 4). These results substantiate the DRIP150–GR AF-1 interaction observed in the yeast two-hybrid assay, and indicate that DRIP150 interacts with a functional GR AF-1 surface.

Differential effects of TSG101 and DRIP150 on GR-dependent transcriptional activation

Because TSG101 and DRIP150 require a competent GR AF-1 for interaction, we anticipated that these factors would play a role in GR-dependent transcriptional regulation. The effect of full-length TSG101 expression on GR-dependent transcriptional activation was examined in yeast. Yeast were transformed with expression vectors encoding GR AF-1 derivatives fused to the LexA DNA

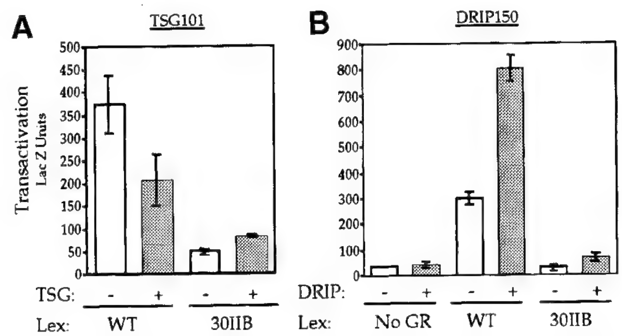

Fig. 5. TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in yeast. $TSG101_{1-381}$ (**A**) or $DRIP150_{40-1454}$ (**B**), under the control of a galactose-inducible promoter, were transformed into yeast expressing $GR_{107-237}$wt or 30IIB fused to the LexA DNA binding domain along with a Lex-operator-linked β-galactosidase reporter gene. Transcriptional activity is determined by quantitative liquid β-galactosidase assays in the presence and absence of TSG101 or DRIP150 after a 12 h incubation in galactose- or glucose-containing media, as indicated, at 30°C. β-galactosidase activity shown represents the average from four independent colonies and the standard deviation is shown.

binding domain and TSG101 under the control of a galactose-inducible promoter or the vector alone, along with a Lex-operator-linked β-galactosidase reporter plasmid. As shown in Figure 5A, GR AF-1 transcriptional activation is reduced in the presence of TSG101. These results suggest that TSG101 functions as a transcriptional repressor that binds to and inhibits GR AF-1-dependent transcriptional activation.

To assess the effect of DRIP150 expression on GR transcriptional activation, another set of yeast strains was created that contain the GR derivatives fused to the LexA DNA binding domain, along with a near full-length DRIP150 derivative ($DRIP_{40-1454}$) under the control of a galactose-inducible promoter or the vector alone, and a Lex-operator-linked reporter gene. Expression of DRIP150 in yeast enhanced transcriptional activation by wtGR nearly 3-fold (Figure 5B). In contrast, co-expression of DRIP150 had little effect on 30IIB, indicating that activation of GR AF-1 by DRIP150, as is its ability to interact, is dependent upon the integrity of the GR AF-1 activation surface. Thus, DRIP150 increases GR AF-1-dependent transcriptional activation in yeast, suggesting that DRIP150 represents a novel GR AF-1 coactivator.

To assess whether TSG101 and DRIP150 overexpression affects GR-dependent transcriptional activation in mammalian cells, we transiently transfected HeLa cells, containing endogenous GR, with increasing concentrations of expression vectors encoding either TSG101 or DRIP150 along with a mouse mammary tumour virus (MMTV)-luciferase reporter gene. Overexpression of TSG101 repressed GR-dependent transcriptional activation from the MMTV promoter in a dose-dependent manner, while increasing amounts of DRIP150 resulted in enhanced transcriptional activation by GR (Figure 6A and B). The effect of DRIP150 on GR was not restricted to a single cell type, since overexpression of DRIP150 in CV-1 cells also results in a dose-dependent increase in GR transcriptional enhancement (Figure 6C). Thus, TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in mammalian cells.

We next assessed whether a TSG101 derivative lacking

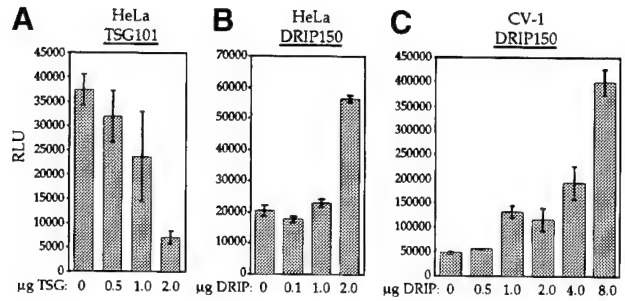

Fig. 6. TSG101 represses, whereas DRIP150 enhances, GR-dependent transcriptional activation in mammalian cells. HeLa cells were transfected with expression plasmids for full-length TSG101 (**A**) or full-length DRIP150 (**B**), at the indicated concentrations along with the GR-responsive, MMTV-Luc reporter plasmid and the total amount of DNA per dish was equalized with an empty expression vector. CV-1 cells were transfected with DRIP150 (**C**) at the indicated concentrations along with a GR expression vector and an MMTV-Luc reporter plasmid. Adding empty expression vector equalized the total amount of DNA per dish. Cells were treated with 100 nM dexamethasone for 12 h and GR transcriptional activity was assayed as described in Materials and methods, normalized to protein concentration and expressed as relative luminescence units (RLU). The average of three independent experiments is shown with the standard deviation.

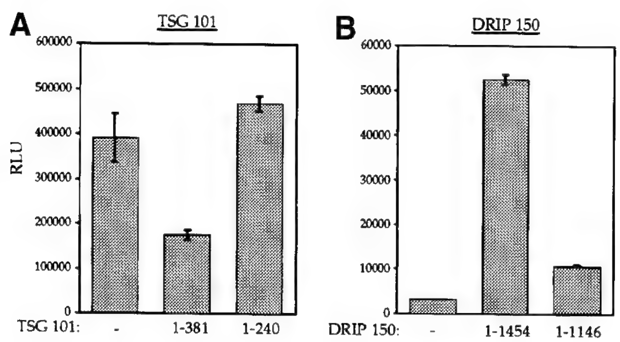

Fig. 7. The effects of TSG101 and DRIP150 on GR transcriptional regulation are dependent on the GR-interacting regions. (**A**) Repression of GR transactivation is associated with the TSG101 GR-interacting region. HeLa cells were transfected with GR_{1-556}, a constitutively active GR derivative lacking the LBD, and either 1.5 μg of the empty expression vector (–), full-length $TSG101_{1-381}$ (1.5 μg) or a C-terminal deletion of $TSG101_{1-240}$ (1.5 μg) that does not interact with GR. (**B**) Enhancement of GR transactivation is associated with the DRIP150 GR-interacting region. HeLa cells were transfected with GR_{1-556} and either an empty expression vector (1 μg), full-length $DRIP150_{1-1454}$ (1 μg) or a C-terminal deletion $DRIP150_{1-1146}$ (1 μg) derivative, and GR activity was determined as described in Figure 6. The average of three independent experiments is shown with the standard deviation.

the GR-AF-1-interacting region was capable of affecting GR-dependent transcriptional activation. HeLa cells were transfected with an MMTV-luciferase reporter gene along with either an empty expression vector, full-length TSG101 (1–381) or a C-terminal deletion of TSG101 (1–241) incapable of interacting with GR AF-1 (see Figure 3). Full-length TSG101 is capable of repressing GR transcriptional activation, whereas the C-terminal TSG101 truncation is not (Figure 7A). Therefore, the inhibition of GR transcriptional activation by TSG101 requires the TSG101 GR-interacting region.

We also examined whether a DRIP150 derivative lacking the GR-AF-1-interacting region was able to increase GR transactivation. Full-length DRIP150 (1–1454) or a C-terminal deletion derivative, DRIP150 (1–1146), lacking the GR-interacting region was transfected into HeLa cells and GR-AF-1-dependent transcriptional activation was measured. Transcriptional activation of GR is increased 10-fold in the presence of full-length DRIP150 overexpression, whereas the DRIP150 (1–1146) derivative lacking the interacting region elicits an ~2-fold increase in GR enhancement (Figure 7B). These results indicate that the enhanced GR transactivation observed upon DRIP150 overexpression is largely dependent upon the DRIP150 GR-interacting region.

DRIP205 interacts with GR LBD in a hormone-dependent manner

Since association of the DRIP complex with NRs was originally discovered as a ligand- and AF-2-dependent process through a single subunit (Rachez *et al.*, 1999), DRIP205, we also examined whether DRIP205 is capable of interacting with the GR ligand binding domain (LBD) and whether this interaction is hormone dependent. We *in vitro* transcribed and translated full-length GR, as well as GR N- and C-terminal deletion derivatives (Figure 8A), and precipitated them using GST–DRIP205 in the absence and presence of dexamethasone. Remarkably, DRIP205 bound full-length GR in a ligand-dependent manner (Figure 8B). GR LBD (GR-C) also efficiently associated with DRIP205 in the presence but not the absence of dexamethasone. In contrast, the GR N-terminus (GR-N), the target of DRIP150 association, did not interact with DRIP205, indicating that the GR LBD is sufficient for ligand-dependent interaction with DRIP205.

Interestingly, *in vitro*-translated full-length GR bound GST–DRIP150 in a dexamethasone-independent fashion, consistent with DRIP150's interaction with the glucocorticoid-independent AF-1 (Figure 8C). GR LBD failed to associate with GST–DRIP150 either in the presence or absence of dexamethasone (Figure 8C). These results establish that DRIP150 interacts with GR AF-1, but not AF-2. Together, our data indicate that DRIP205 associates with GR LBD in a hormone-dependent manner, whereas DRIP150 interacts with GR AF-1, thereby linking AF-1 to AF-2 via the DRIP coactivator complex.

To assess whether DRIP205 and DRIP150 overexpression affects GR-dependent transcriptional activation, we transiently transfected HeLa cells with expression vectors encoding either DRIP205 or DRIP150, individually or together, and an MMTV-luciferase reporter gene. Expression of DRIP205 had little effect on GR transcriptional activation, whereas expression of DRIP150, under the conditions examined, increased receptor-dependent transcriptional activity ~10-fold (Figure 8D). Co-expression of DRIP205 and DRIP150 further augmented GR-dependent transactivation 20-fold, lending support to the idea that components of the DRIP coactivator complex are key regulators of GR transcriptional enhancement.

Discussion

We have identified TSG101 and DRIP150 as proteins that interact with GR AF-1 and differentially affect GR transcriptional activation. TSG101 expression results in a reduction of GR transcriptional activation in both yeast and mammalian cells, whereas overexpression of DRIP150 increases GR transcriptional enhancement in these systems.

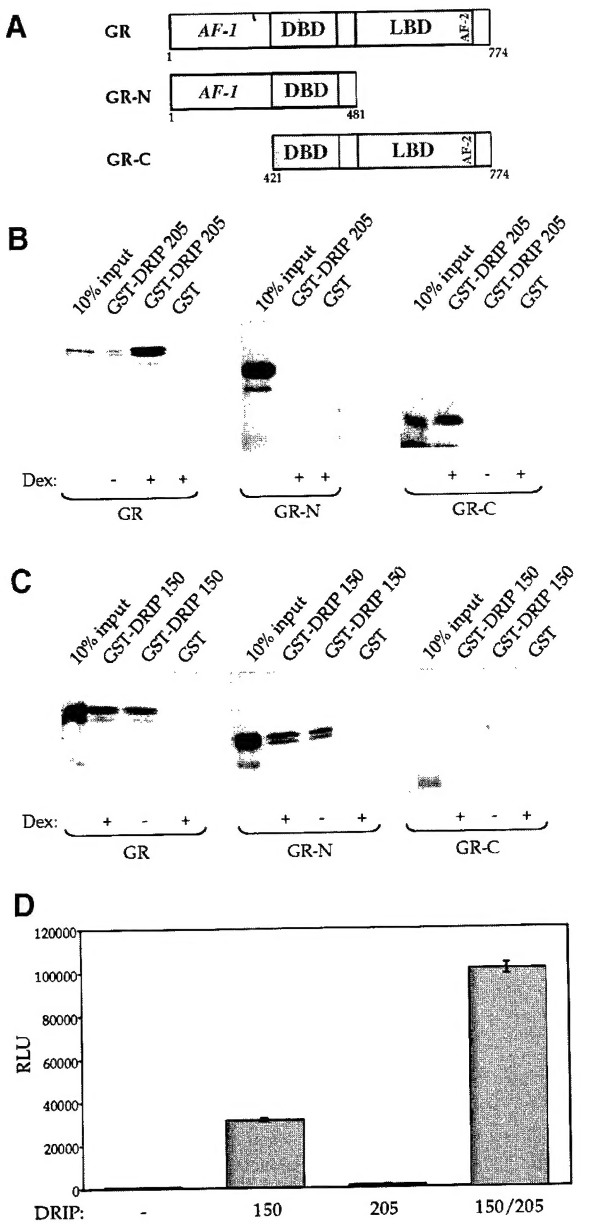

Fig. 8. The GR N- and C-termini associate with DRIP150 and DRIP205, respectively. (**A**) Schematic representation of full-length human GR_{1-774} (GR), GR_{1-481} (GR-N) and $GR_{421-774}$ (GR-C) derivatives. (**B**) Ligand-dependent interaction of DRIP205 with GR derivatives. (**B**) Ligand-dependent interaction of DRIP205 with GR *in vitro*. To examine GR–DRIP205 interaction, full-length GR was transcribed and translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–DRIP205, in the absence or presence of dexamethasone, or GST. The GR-N and GR-C derivatives were translated *in vitro* and incubated with Sepharose beads containing bound GST–DRIP205, in the absence or presence of dexamethasone, or GST. Bound GR was resolved by SDS–PAGE and visualized by autoradiography. Note the hormone-dependent interaction of GR and GR-C with DRIP205. (**C**) Ligand-independent association of DRIP150 with the GR *in vitro*. GR, GR-N or GR-C were translated *in vitro* in the presence of [^{35}S]methionine (lane 1; 10% input) and incubated with Sepharose beads containing bound GST–DRIP150 in the absence or presence of dexamethasone, or GST. Bound GR was resolved by SDS–PAGE and visualized by autoradiography. Note the ligand-independent interaction of GR and GR-N with DRIP150. (**D**) Enhancement of GR transactivation by DRIP150 and DRIP205. HeLa cells were transfected with either 3.0 μg of the empty expression vector (–), DRIP150 (1.5 μg), DRIP205 (1.5 μg) or DRIP150 (1.5 μg) and DRIP205 (1.5 μg) and assayed as described in Figure 6, and GR transactivation was measured and expressed as RLU.

The differential effects of TSG101 and DRIP150 on GR transcriptional activation are dependent upon the DRIP150 and TSG101 GR-interacting regions, since deletion of these domains renders both cofactors less capable of affecting GR transcriptional activation. Thus, DRIP150 and TSG101 represent a new class of GR AF-1-associated cofactors that regulate receptor transcriptional activation in a reciprocal manner. To our knowledge, this is the first example of steroid receptor association with DRIPs.

We also demonstrate a ligand-dependent interaction between DRIP205 and GR LBD. Although overexpression of DRIP205 had little effect on GR transcriptional enhancement, co-expression of DRIP150 and DRIP205 augments GR transcriptional activation to a degree greater than either molecule alone, suggesting that DRIP205 and DRIP150 cooperate in promoting GR transcriptional activation. Thus, DRIP150 and DRIP205 associate with GR N- and C-termini, respectively, functionally linking GR AF-1 to AF-2.

TSG101 was originally identified in a screen for genes that, when inactivated, promote neoplastic transformation (Li and Cohen, 1996). Our results suggest that TSG101 functions as a transcriptional repressor protein, decreasing GR-dependent transcriptional activation by binding and repressing AF-1. TSG101 also interacts with the AF-1 regions of AR and TR, and expression of TSG101 decreases AR-dependent transcriptional activation (not shown). Although Watanabe *et al.* (1998) demonstrated previously that TSG101 suppresses the transcriptional activity of a variety of NRs, including GR, AR and TR, our findings link TSG101 specifically to AF-1 domains in the receptors.

The ability of TSG101 to bind to GR AF-1 and inhibit transactivation is reminiscent of the interaction between another tumor susceptibility gene, Rb, and the transcription factor it negatively regulates, E2F-1. Rb is capable of recruiting a histone deacetylase (HDAC) to E2F-1, thereby repressing E2F-regulated promoters (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Conceivably, TSG101 functions in an analogous fashion, recruiting a HDAC to GR and repressing its transcriptional activity through changes in chromatin structure. This idea is also consistent with the ability of TSG101 to repress a wide variety of activators and promoters when overexpressed. Additional experiments will be required to determine the mechanism of TSG101-mediated suppression of GR transactivation and whether it is mediated by HDACs.

DRIP150 was initially isolated as a subunit of a multiprotein complex that interacts in a ligand-dependent manner with the AF-2 domain of VDR and enhances VDR transcriptional activation, indicating that the complex may have evolved to link NRs to the transcription apparatus. Recently, DRIP150 has been identified as a component of several multiprotein complexes, including ARC, CRSP, TRAP/SMCC and NAT, that regulate other transcriptional activators, suggesting a broader role for the DRIP complex in transcriptional regulation (Sun *et al.*, 1998; Näär *et al.*, 1999; Rachez *et al.*, 1999; Ryu *et al.*, 1999). In both yeast and mammalian cells, DRIP150 is capable of increasing GR-mediated transcriptional activation when overexpressed. DRIP205 binds directly to the LBDs of several NRs in a ligand-dependent manner. Here we demonstrate that GR LBD also interacts with DRIP205 in

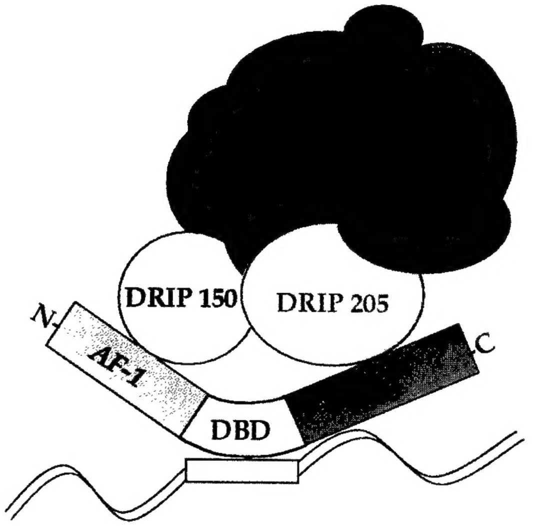

Fig. 9. A model for the regulation of GR by DRIP150 and DRIP205. Shown schematically is the full-length GR, which recruits DRIP150 (light blue oval) and DRIP205 (yellow oval) through an association with the GR N-terminus (green rectangle) and C-terminus (orange rectangle), respectively, thereby facilitating GR transactivation. The other subunits of the heteromeric DRIP complex are also depicted (dark blue ovals); however, their precise molecular interactions and effects on GR-mediated transactivation have not been determined.

a hormone-dependent manner. Although expression of DRIP205 has little effect on GR-mediated transcriptional activation, it appears to synergize with DRIP150 to facilitate GR-mediated transcriptional activation. From these results, we propose a model whereby DRIP150 and DRIP205 serve as a molecular bridge functionally linking the GR N- and C-terminal transcriptional activation functions, AF-1 and AF-2, thus facilitating transcriptional activation (Figure 9). Consistent with this view, recent findings from our laboratory suggest that distinct coactivators may promote the productive interaction between the GR N-terminus and LBD to facilitate GR transcriptional activation of the endogenous p21 promoter in SAOS2 cells (Rogatsky *et al.*, 1999). Although the N- and C-terminal regulatory regions of the estrogen receptor function synergistically to activate transcription via SRC1 (McInerney *et al.*, 1996), this is the first example whereby two members of the heteromeric DRIP coactivator complex, DRIP150 and DRIP205, associate with distinct GR domains to facilitate transcriptional enhancement. It is likely that the DRIP complex or subcomplexes containing DRIP150 and DRIP205 are important mediators of GR transcriptional activation. It is also conceivable that differences in the ratios of DRIP150 to DRIP205 within cells will contribute to cell- and enhancer-specific regulation by the GR. Thus, the ability of GR to target multiple proteins within the DRIP coactivator complex via distinct receptor domains likely represents a general and versatile means of regulating gene expression.

Materials and methods

Construction of plasmids

Yeast expression vectors for the LexA–GR fusion proteins (Lex–$GR_{107-237}$wt and Lex–$GR_{107-237}$30IIB) were created by digesting the respective full-length rat GR clones with *Nco*I, filling in the 5′ overhangs with DNA polymerase Klenow fragment to create a blunt end, followed by *Bgl*II digestion and subcloning the fragments into pEG202 digested with *Eco*RI, the 5′ end filled in, and *Bam*HI. Yeast two-hybrid 'bait' proteins, B42–$GR_{107-237}$wt and –30IIB, were constructed by subcloning GR *Eco*RI–*Xho*I fragments from pEG202:$GR_{107-237}$, wt and 30IIB, into the corresponding sites in pJG4-5. The LexA–HeLa cell 'prey' library was constructed by subcloning *Eco*RI–*Xho*I fragments from a pJG4-5:HeLa cell library into the pEG202 digested with *Eco*RI–*Xho*I. The LexA–TSG101 fusion protein was created by digesting the full-length TSG101 (a gift from S.Cohen, Stanford University, CA) with *Pvu*II, and subcloning the fragment into the *Bam*HI site of pEG202 also made blunt by filling in its 5′ overhang. An *Eco*RI–*Xho*I fragment from pEG202 was then subcloned into pJG4-6 to create a HA-tagged version of full-length TSG101. The TSG101 C-terminal truncations 1–82, 1–121, 1–206, 1–240 and 1–339 were created by digesting LexA–TSG101 with *Spe*I, *Bsp*HI, *Ava*II, *Ban*I or *Hin*dIII, respectively, filling in their 5′ overhangs with Klenow, digesting with *Eco*RI and subcloning into pEG202 digested with *Nco*I, filled in, and *Eco*RI. The mammalian expression vector pCDNA3:FLAG–TSG101 was created by subcloning the FLAG–TSG101 *Pvu*II fragment into pCDNA3 digested with *Bam*HI with its 5′ overhang filled in. pEG202:$DRIP150_{40-1454}$ (Lex–DRIP) and pJG4-6:$DRIP150_{40-1454}$ (HA–DRIP) were created by subcloning a *Not*I fragment (filled-in blunt) from pCDNA3:DRIP150 into pEG202 and pJG 4-6 digested with *Eco*RI–*Xho*I (filled-in blunt). pCDNA3:DRIP150(1–1146) was created by digesting pCDNA3:DRIP150 with *Nco*I, filling in the 5′ overhang, digesting with *Eco*RI and subcloning the fragment into *Eco*RI–*Xho*I (filled-in blunt) pCDNA3. pJG4-5: Sp-$1A_{83-262}$, pJG4-5:Sp-$1B_{263-542}$, pJG 4-5:TAF $130_{270-700}$, pJG 4-5: $VP16_{412-456}$ and pJG4-5:CREB-N_{3-296} were provided by N.Tanese (New York University School of Medicine, New York). pJG4-5: SRC-$1_{374-800}$ and pJG4-5:T3R$\beta2_{1-159}$ were provided by H.Samuels (New York University School of Medicine, New York). pJG4-5:AR_{18-500} was provided by S.Taneja (New York University School of Medicine, New York). The pJK103 reporter plasmid, which contains a single LexA operator linked to β-galactosidase, was used in all activity assays of the LexA fusion proteins and in the modified yeast two-hybrid assay. The pCMV:wtGR expression plasmid was used to produce rat GR, pMMTV:luciferase reporter was used to assay GR transcriptional activity, while pCMV:LacZ constitutively expresses β-galactosidase, a marker for transfection efficiency.

Modified yeast two-hybrid approach

The modified yeast two-hybrid approach is based on the LexA system as described in Bartel and Fields (1997) with certain modifications. EGY188 (*trp1 his3 ura3 leu2*) contains a chromosomally integrated leucine (*LEU*) reporter gene driven by a single LexA operator (*leu2::2* LexAop-*LEU2*), allowing for the selection of interacting proteins on galactose, leucine-deficient plates. EGY188 was transformed by the lithium acetate method (Gietz, 1995) with (i) pJG4-5:$GR_{107-237}$ bait, (ii) pEG202:HeLa cell cDNA library and (iii) pJK103, a β-galactosidase reporter gene with a single LexA operator. pJG 4-5, which contains the GAL1-10 promoter, expresses B42 activation domain fusion proteins grown in the presence of galactose, while pEG202, driven by the alcohol dehydrogenase promoter, constitutively expresses LexA DNA binding domain fusion proteins. Potential interacting proteins were selected by plating the cDNA library-expressing transformants onto galactose plates lacking leucine and containing the chromogenic substrate X-gal. As a majority of the colonies contained cDNAs that encode an activation domain (self-activating false positives) rather than a GR-interacting protein, a second screen was used. Colonies that grew on galactose in the absence of leucine and expressed LacZ (i.e. blue) were replica-plated onto glucose-X-gal plates. Since the expression of GR is under the control of the galactose-inducible, glucose-repressible Gal1-10 promoter, potential interactors are blue on galactose–X-gal plates, conditions where the GR bait is expressed, but white on glucose–X-gal plates. In contrast, false positives are blue on both glucose–X-gal and galactose–X-gal-plates, independent of GR expression, and were not analyzed.

Quantitative liquid β-galactosidase assay

Yeast were grown in selective liquid media containing 2% glucose for 10 h, pelleted, washed once in H_2O, normalized according to cell number and resuspended to an optical density (OD 600) of 0.15 in either 2% glucose or 2% galactose/1% raffinose, depending on the experiment. β-galactosidase assays were performed 12 h later as described previously (Garabedian, 1993).

GST pull-down assays

GST fusion proteins (20 μg), GR $wt_{107-318}$, GR $30IIB_{107-318}$, $DRIP150_{1345-1493}$ and $DRIP205_{527-774}$ or GST, immobilized on glutathione–Sepharose beads were preincubated in binding buffer [20 mM Tris pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.05% Nonidet P-40 (NP-40), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 4 mg/ml bovine serum albumin (BSA)] for 30 min at 4°C. *In vitro*-translated, [^{35}S]methionine-labeled (Promega TNT reticulocyte lysate system) DRIP150 or GR was incubated with the immobilized fusion proteins for 1 h at 4°C. The beads were washed four times in wash buffer (20 mM Tris pH 7.9, 170 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.1% NP-40, 0.1 mM PMSF and 1 mM DTT), resuspended in 2× SDS sample buffer and boiled for 3 min; the associated proteins were resolved by SDS–PAGE and visualized by autoradiography.

Mammalian cell culture and transient transfection assays

Human cervical carcinoma cell lines (HeLa) and African green monkey kidney cells (CV-1) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT), 50 U/ml each of penicillin and streptomycin (Life Technologies) and 2 mM L-glutamine (Life Technologies). For transfections, HeLa cells were seeded in 35 mm dishes at a density of 1.5×10^5, washed once with serum-free medium and transfected with 0.1 μg MMTV-Luc, 0.05 μg pCMV-LacZ, and the indicated concentrations of pCDNA3:FLAG-TSG101, pCDNA3:DRIP150 and pCDNA3:DRIP205, using 5 μl of lipofectamine reagent (Life Technologies) in a total volume of 1 ml of serum-free, phenol red-free DMEM per 35 mm dish according to the manufacturer's instructions. Three hours post-transfection, the transfection mix was removed, the cells were refed with 2 ml of DMEM–10% FBS, allowed to recover for 5–7 h, and were fed again with fresh DMEM–10% FBS supplemented with 100 nM Dex or an identical volume of 100% ethanol and incubated for 12 h. Transfected cells were washed twice in phosphate-buffered saline and harvested in 1× reporter lysis buffer (Promega) as per the manufacturer's instructions. CV-1 cells were transfected with 0.5 μg pCMV:GR, 1 μg MMTV-Luc, 0.5 μg pCMV-LacZ and the indicated concentration of pCDNA3:DRIP150 by the calcium phosphate method as described previously (Lemon and Freedman, 1996). Luciferase activity was quantified in a reaction mixture containing 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 1 mM ATP, 0.1 mg/ml BSA, 1 mM DTT, using a Lumen LB 9507 luminometer (EG&G Berthold) and 1 mM D-luciferin (Analytical Luminescence Laboratory) as substrate. Lysates from the transfected cells were also assayed for β-galactosidase activity as described elsewhere (Alam and Cook, 1990).

Immunoblotting

Yeast protein extracts were prepared from 2 ml cultures and lysed using glass beads as previously described (Knoblauch and Garabedian, 1999). Extracts were normalized according to the Bradford protein assay (Bio-Rad) and separated on either 10 or 4–20% SDS–polyacrylamide gels (Novex) and transferred to Immobilon paper (Millipore). Membranes were probed with polyclonal antibodies for α-LexA (a gift from E.Golemis) or monoclonal antibodies to HA (12CA5; Boehringer Mannheim). The blots were developed using horseradish peroxidase-coupled donkey anti-rabbit or sheep anti-mouse antibodies and the enhanced chemiluminescence (ECL) substrate as per the manufacturer's instructions (Amersham–Pharmacia).

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